Development of genetically encoded cell type-specific activity markers for the mouse olfactory system

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I owe a great deal to my family, my amma, achan, and sisters for encouraging me and making me what I am ….

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Abstract

The olfactory system consists of the olfactory epithelium in the nasal cavity, the olfactory bulb, the olfactory cortex and higher brain regions. Olfactory information processing begins at the olfactory sensory neurons that express odorant receptors. These proteins bind odors and transduce a biochemical signal that is converted to action potentials by the neuron. In the olfactory bulb, sensory neurons synapse with projection neurons called the mitral/tufted cells in structures called glomeruli and pass on information to the olfactory cortex. The olfactory bulb is not a simple way station for transmission of odorant information; significant processing of information is carried out by the circuitry in the olfactory bulb. The major neuronal components of the bulb that take part in these interactions are the periglomerular cells, short axon cells and the granule cells. Neural information processing is carried out by ensembles of neurons and it is important to study such populations to reach an understanding of nervous system function. Recent developments in identification of cell type specific promoters and construction of GFP based sensors of Ca^{2+} influx and voltage changes has made it possible to devise genetic strategies to study them. Another strategy that is frequently employed is studying the expression of Immediate Early Genes, especially *C-fos* after stimulation. In this study we developed two mice strains, one that can express the short half-life version of EYFP called d2EYFP under the *C-fos* promoter and another that codes for the calcium sensitive fluorescent protein ratiometric pericam. The expression of both these reporters can be made cell type specific because they are blocked by a floxed stop cassette and can be activated in specific cell types by crossing to mice strains that express the cre recombinase under specific promoters. A Bacterial Artificial Chromosome that carries the *C-fos* gene was modified by inserting a STOP-IRES-d2EYFP cassette at the *C-fos* locus after truncating the *C-fos-coding* region. The loxP site in the BAC's back bone was removed by another round of recombination and circular BAC molecules were used of pronuclear microinjection. One positive founder was identified and is being bred for

further analysis. Ratiomeric pericam was cloned under the ubiquitous CAG promoter with a stop cassette in between and transgenic mice were made after testing expression in cell culture. Seven different transgenic lines with different transgene copy numbers were obtained and were subjected to further analysis. No transgene expression could be detected upon breeding to a CMV-Cre mouse strain that should delete in all cells. Analysis of DNA of double transgenic mice showed no deletion of stop cassette. The mice were further crossed to a CamKIIα-cre strain. Pericam was detected in fore brain of double transgenics by western blotting with anti-calmodulin and anti-GFP antibodies. Immunohistochemistry of olfactory bulb and olfactory epithelium revealed high level expression in patches of olfactory epithelium and much lower expression in granule cells of olfactory bulb. Further experiments are under way to characterize both the strains and to start optical imaging in the olfactory bulb and olfactory epithelium.

Zusammenfassung

Das Geruchssystem besteht aus dem Riechepithel in der Nasenhöhle, dem Bulbus olfaktorius, dem olfaktorischen Cortex und weiteren Gehirnregionen. Die Informationsverarbeitung im olfaktorischen System beginnt mit den Geruchsrezeptorneuronen, die die Geruchsrezeptorgene exprimieren. Diese Rezeptoren binden Geruchsstoffe und generieren so ein biochemisches Signal, das zu Aktionspotentialen umgewandelt wird. Im Bulbus olfaktorius bilden die sensorischen Neuronen Synapsen mit den Projektionsneuronen in Neuropilknäueln, den sogenannten Glomeruli. Die Projektionsneuronen, Mitral- und Büschelzellen tragen die Information zum Riechcortex. Der Bulbus olfaktorius ist nicht nur eine einfache Relaisstation, hier findet schon Informationsverarbeitung statt durch periglomeruläre Zellen, Short-Axon-Zellen, und Körnerzellen. In allen diesen Interaktionen sind Zellensembles beteiligt, und es ist daher wichtig, die Antworteigenschaften von Populationen von Neuronen zu untersuchen, um das Geruchssystem zu verstehen.

Seit kurzem sind genetisch kodierte Calcium-Indikatoren verfügbar geworden, die zusammen mit der Entwicklung zelltypspezifischer Promotoren es erlauben sollten, die neuronale Aktivität auf der Populationsebene zu untersuchen. Solche Promotoren sollten es auch ermöglichen, die Aktivierung von Immediate-Early Genen wie *C-fos* (welche die neuronale Aktivierung wiederspiegelt) zelltypspezifisch zu charakterisieren.

In dieser Dissertation habe ich zwei Mausstämme generiert, in einem wird die destabilisierte Version von EYFP, d2EYFP, unter der Kontrolle des *C-fos* Promotors exprimiert, im anderen wird der Calciumindikator ratiometrisches Pericam von einem ubiquitären Promotor, CAG, exprimiert. Nichtsdestotrotz kann die Expression in beiden Fällen zelltypspezifisch geführt werden, da eine loxP-STOP-loxP Kassette vorgeschaltet ist, und durch Kreuzung mit einer geeigneten CRE-Rekombinase-exprimierenden Mauslinie entfernt werden kann.

Ein bakterielles artifizielles Chromosom (BAC) mit dem *C-fos* Gen wurde mittels homologer Rekombination durch Einbringung einer STOP-IRES-d2EYFP Kassette so modifiziert, dass nur ein trunkiertes *C-fos* entstehen kann. Nach Eliminierung einer loxP Sequenz im BAC-Vektor wurden intakte modifizierte BAC Moleküle mittels pronukleärer Injektion in Mausoozyten engebracht. In drei Injektionsrunden konnte eine Integration in die Keimbahn festgestellt werden, diese Maus wird gegenwärtig für weitere Analysen weitergezüchtet. Das Konstrukt mit dem Calcium-Indikatorgen wurde als klassisches Transgen konzipiert und in Zellkulturexperimenten auf die erwartete Funktionsweise überprüft. Nach pronukleärer Injektion wurden sieben verschiedene Mauslinien erhalten. Die Überprüfung einiger dieser Linien durch Kreuzung mit einer CMV-Cre Mauslinie ergab unerwarteterweise keine Aktivierung des Indikatorgens. In der Kreuzung mit einer CamKIIalpha-Cre Mauslinie konnten in zwei dieser Linien Bestandteile des Calcium-Indikators im Vorderhirn mittels Western Blot nachgewiesen werden; Calmodulin über einen spezifischen Antikörper und Pericam über einen GFP-Antikörper, der auch Pericam erkennt. Präliminäre immunhistochemische Experimente im Bulbus olfaktorius und im Riechepithel zeigten starke, wenngleich inhomogene Expression in Regionen des Riechepithels und möglicherweise eine schwache Expression in der Körnerzellschicht des Bulbus olfaktorius. Experimente zur weiteren Charakterisierung dieser zwei Linien sind begonnen worden.

I.INTRODUCTION

1. Olfaction

Olfaction is an important sense for animals. From an evolutionary standpoint, it is an ancient sense. It is used in finding food and mate, kin recognition and many social behaviors and provides sensual pleasures (flowers, perfumes) and warnings (spoiled food, harmful chemicals). Odor related physiology can cause immediate changes in the affective state and arousal level in humans and evoke recall of emotional valence and personal experiences related to the odor (proustian phenomena). Human beings, considered microsmates, can distinguish about ten thousand odors while macrosmates like mice are thought to be able to distinguish many more. Olfactory research today encompasses many levels from the molecular through the cellular and systemic to the behavioral, and many species (Shipley and Ennis, 1996). This thesis describes research on the mammalian olfactory system, specifically, that of the mouse, using a molecular-systemic approach.

1.1 Organization of the mammalian olfactory system

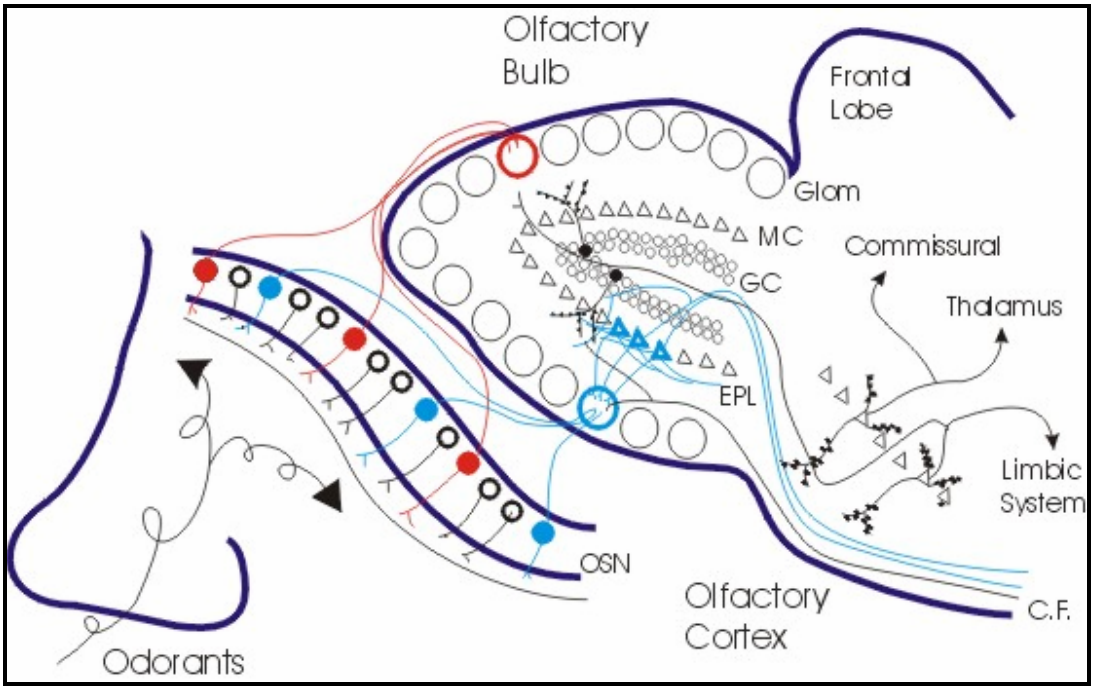

Fig I.1 Mammalian olfactory system. OSN, olfactory sensory neuron; Glom, glomeruli; MC, mitral cell; GC, Granule cell; EPL, External plexiform layer; CF, centrifugal fibres. From: http://neurowww.cwru.edu/faculty/Strowbridge/

A schematic diagram of the human olfactory system is shown in fig I.1. Mammals have a highly developed olfactory system that is distributed among the peripheral and central nervous systems. In the nasal cavity is a special kind of epithelium, the olfactory epithelium, consisting of supporting cells, mature olfactory sensory neurons and neuronal progenitor cells. Of these, the bipolar olfactory sensory neurons are involved in odorant binding and signaling. They consist of a single dendrite terminating in a tuft of cilia, which lines the epithelium, a cell body and a long axon that projects to the olfactory bulb through the cribriform plate. The olfactory bulb is a laminar structure, which is involved in processing olfactory information before it is transmitted to the higher brain regions, among them the olfactory cortex. The superficial layer of the olfactory bulb is called the olfactory nerve layer (ONL). It is made of axons from the olfactory neurons and glial cells. Immediately below the ONL is the glomerular layer. It consists of spherical or ovoid neuropils of about 100 to 150 micrometer diameter of which there are about 2000 per bulb in mouse. In the glomeruli the OSN axons mingle with axons and dendrites of juxtaglomerular cells and mitral cells. The secondary dendrites of mitral cells and granule cells form the external plexiform layer that lies below the glomerular layer. The mitral cell bodies themselves form the next layer, the mitral cell layer. From each mitral cell a primary dendrite penetrates a glomerulus. Each glomerulus is connected to about 25 mitral cells in mouse. Mitral cells along with tufted cells are the primary output or projection neurons, cells that take information to higher brain regions. The innermost is the granule cell layer made up of cell bodies of anaxonal granule cells, which gives way to the sub-ependymal zone made of cells that line the ventricle. Some progenitors of bulbar neurons are found here.

1.2 The odorant receptors recognize odorants

The molecular era in olfaction began with the cloning by RT-PCR of putative odorant receptors that belong to the class of membrane proteins called GPCRs (G-protein coupled receptors)(Buck and Axel, 1991). Information was available that there existed a GTP dependent adenylase cyclase activity in olfactory cilia. This pointed to the possibility that the mechanism of odor recognition could involve GPCRs expressed in the receptor neurons (Reed et al., 1992). Based on this information, Buck and Axel produced degenerate oligonucleotide primers against conserved domains of GPCRs and cloned them from olfactory epithelium mRNA. The full-length products when assembled from these partial cDNAs turned out to be typical GPCRs with seven membrane spanning loops. They are abundantly expressed in the cilia of mature OSNs and have been shown to bind odorants. In mice there are about 1300 odorant receptor genes (about 20% of which are pseudogenes), making them the largest among the gene families (Niimura and Nei, 2005). None of these proteins have been crystallized for X-ray crystallographic structure determination, but molecular modeling studies based on the structure of rhodopsin have been done. From these and other studies, it has been inferred that the odorant-binding pocket reside between transmembrane regions three, four and five(Luu et al., 2004). An active area of research is finding receptor-ligand pairs by functionally expressing the ORs in cell cultures or in the epithelium.

1.3 Odorant induced Signal transduction

The signaling events that occur in the olfactory receptor neurons are topics of intense study (Firestein, 2001). Airborne odorants are swept into the nasal cavity by breathing .The olfactory epithelium is bathed in mucus containing a class of proteins called the odorant binding proteins, which bind and transport odorant molecules to the cilia of the olfactory receptor neurons. The odorant receptors (ORs) non-covalently bind ligands and

initiate signal transduction. As said before, ORs are GPCRs and have trimeric G-proteins (in this case G_{olf}) associated with their cytoplasmic side. Ligand binding results in the exchange of GDP for GTP and dissociation of the α subunit from the βγ subunits. The α sub-unit then binds and activates the enzyme adenylyl cyclase, which converts ATP to cyclic-AMP. Elevated levels of cAMP results in the opening of the cyclic nucleotide gated channel and a consequent influx of calcium ions, which opens an unidentified chloride channel that leads to chloride efflux and depolarization of the neuron. Calcium ions also activate cAMP phosphodiesterase that breaks down cAMP and terminates the signal. Calcium is also generally involved in adaptation, a phenomenon by which continuous exposure to an odor results in insensitivity to that odor. Other second messengers, Inositol tri-Phosphate (IP3) and cGMP, have also been proposed to be involved in signal transduction. Indeed there is a view that there are two different biochemical pathways, one mediated by cAMP and the other by IP3 operating in the ORNs, which could be true, judging from recent evidence. Either way, receptor activation leads to depolarization of the olfactory sensory neuron (generation of EPSP) which produces a train of action potentials relayed to the olfactory bulb.

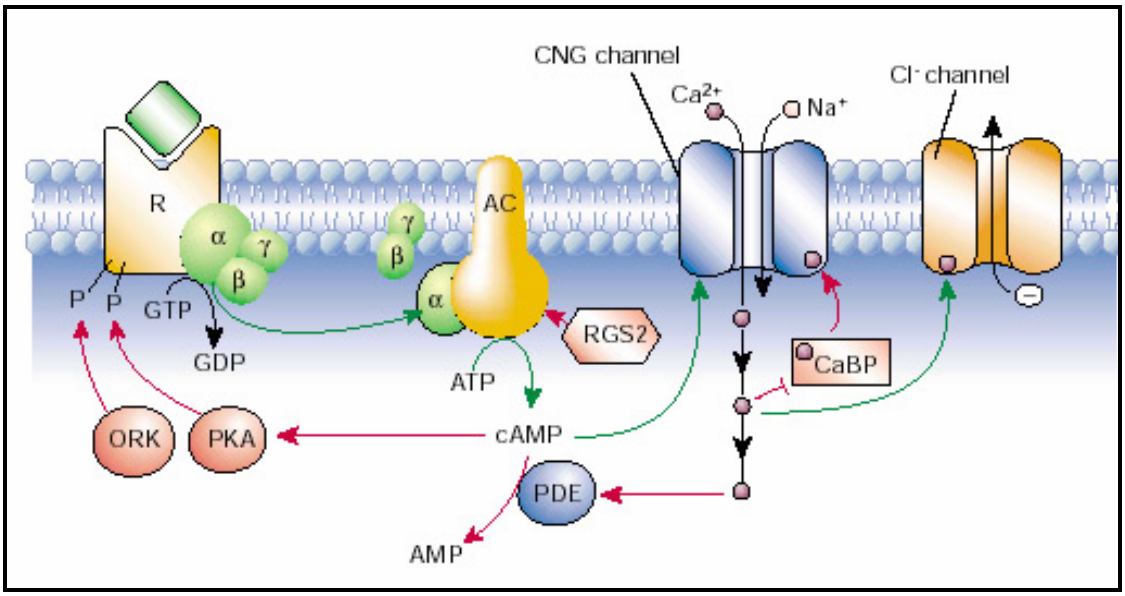

Fig I.2 Sensory transduction in the OSN cilia: AC, adenylyl cyclase; CNG channel, cyclic nucleotide-gated channel; PDE, phosphodiesterase; PKA, protein kinase A; ORK, olfactory receptor kinase; RGS, regulator of G proteins (but here acts on the AC); CaBP,

calmodulin-binding protein. Green arrows indicate stimulatory pathways; red indicates inhibitory (feedback). From: Firestein S Nature. 2001 Sep 13;413(6852):211-8.

1.4 One neuron-one receptor and one receptor-one glomerulus rules

How is the activation of receptors by a particular odorant read out by higher brain regions? The mystery is how the system finds out which receptors are activated by an odor. With in-situ hybridization assays, it was found that each receptor is expressed by only about 0.1% of OSNs, implying that each neuron expresses only one receptor gene. Single cell RT-PCR experiments also suggested the same. Based on these data, Hitoshi Sakano's group in Tokyo raised mice transgenic for a cluster of OR genes in which the MOR 28 gene was tagged with tau-LacZ using Yeast Artificial Chromosomes (YAC) modified in yeast cells. These mice were crossed to gene targeted mice in which the endogenous MOR 28 locus was marked with GFP. In the progeny, no receptor neurons were found that co-expressed LacZ and GFP. This demonstrated that the expression of ORs is mutually exclusive, even for genes with the same sequences and regulatory elements, but differing in chromosomal locus (Serizawa et al., 2000). Thus, the problem of finding which receptor is activated can be simplified to that of finding which set of neurons are activated (Serizawa et al., 2004). In addition, there is allelic exclusion in the system, providing for expression of only one allele from the two present in the chromosomes in a cell (Chess et al., 1994). This possibly eliminates the problem of having receptor proteins with slightly different specificity in the same cell.

Sensory neurons that express different receptors are intermingled in the epithelium. Generally, neighbours of an OR express different receptors, implying that odorants map to the sensory surface in a distributed manner. In-situ hybridization experiments with individual ORs have shown the olfactory epithelium to be divided into four zones, each defined by the set of receptors that are expressed in it (Mori et al., 2000). But, within each zone, cells that express a particular receptor are distributed randomly. Thus, the distribution of ORs is neither receptotopic nor chemotopic in the epithelium. But similar experiments in the olfactory bulb indicated that there might be mRNA of only one OR in

each glomeruli. Building on this observation, Mombearts et al generated mice in which the genomic locus of an odorant receptor, P2, was modified by the addition of IRES-tau-lacZ sequences enabling the transcription of P2-IRES-tau-lacZ bicistronic mRNA. IRES (internal ribosomal entry sequence) element helps in translation of the second cistron, the tau-lacZ, which is an axonically transportable reporter. When the distribution of the P2 neurons were studied by X-Gal staining in these gene targeted mice, it was found that their cell bodies are randomly distributed in the epithelium within the defined zone, but their axons converged to two glomeruli, one lateral and the other medial in the olfactory bulb. Thus, the glomerular layer is highly organized into an array of neuropils each receiving input from neurons expressing only one receptor (Mombaerts et al., 1996). Many studies revealed that each odorant could bind many receptors, thus activating many glomeruli. Thus, each odorant is represented at the level of the olfactory bulb by a unique combination of glomeruli that respond to it. This is called the combinatorial mechanism of odor coding, the central feature of which is that the molecular features of an odorant are converted into a spatial map of neuronal activity. This means that only a few glomeruli are required to code for a particular odorant and that responses overlap. This mechanism has been corroborated by many studies, which are described below. Combinatorial coding has the advantage that thousands of odorants can be mapped using only a few hundred or so receptors and glomeruli. This is in contrast to monospecific coding, which would require a receptor for each odorant. Monospecific coding, but is thought to be used in the pheromone sensing system.

The presence of two glomeruli receiving inputs from the same receptor means that the olfactory bulb contains two mirror-image maps. Also, the co-ordinates of glomeruli within the bulbar surface are rather stereotyped, although not completely invariant. In addition, ORNs expressing closely related odorant receptors (which binds structurally related odorants) project to neighboring glomeruli in the bulb. This is strong indication that the olfactory bulb has a receptotopic and chemotopic organization, contrary to the epithelium.

1.5 Information processing in the olfactory bulb

The patterns seen at the glomerular level are not directly relayed to the higher brain regions. As shown in Fig 1.3, there are many neuronal interactions in the bulb that act upon and modify the glomerular code. The most important of these is the reciprocal dendro-dendritic interaction between mitral cells and granule cells (Yokoi et al., 1995). Activated mitral cells activate granule cells, which in turn repress neighboring mitral cells. This sort of lateral inhibition is thought to sharpen the code by enhancing the contrast between active and inactive regions of the bulb. Another hypothesis is that lateral inhibition serves to synchronize the response patterns of mitral cells and underlie the odor-induced oscillations seen in the olfactory bulb. Mitral cells are also known to be self -inhibitory. In addition, the juxtaglomerular cells, which include the periglomerular cells, external tufted cells and the short axon cells also modify the odor code. Periglomerular cells form dendrodendritic connections between mitral and tufted cells and also axodendritic connections with ORNs which they inhibit. Recently, the short axon cells were shown to mediate center-surround inhibition to create an on center- off surround pattern among neighbouring glomeruli (Aungst et al., 2003). There are also centrifugal feed back projections which arise from virtually all parts of the olfactory nucleus which modify, perhaps by inhibition, the message relayed by the mitral cells. In view of all these, it becomes important to monitor the activity pattern of cells further down stream in the olfactory pathway, namely the mitral and tufted cells and ultimately also the higher brain regions to get a better understanding of the mechanism of olfaction. There are no methods available to monitor the activity of mitral cells at the population level repeatedly, although such methods are available for the OSNs (see below). The only method currently used to study mitral cells is electrophysiology. Although much has been learnt with this method, it is inadequate to see bulb-wide pattern of mitral cell activity.

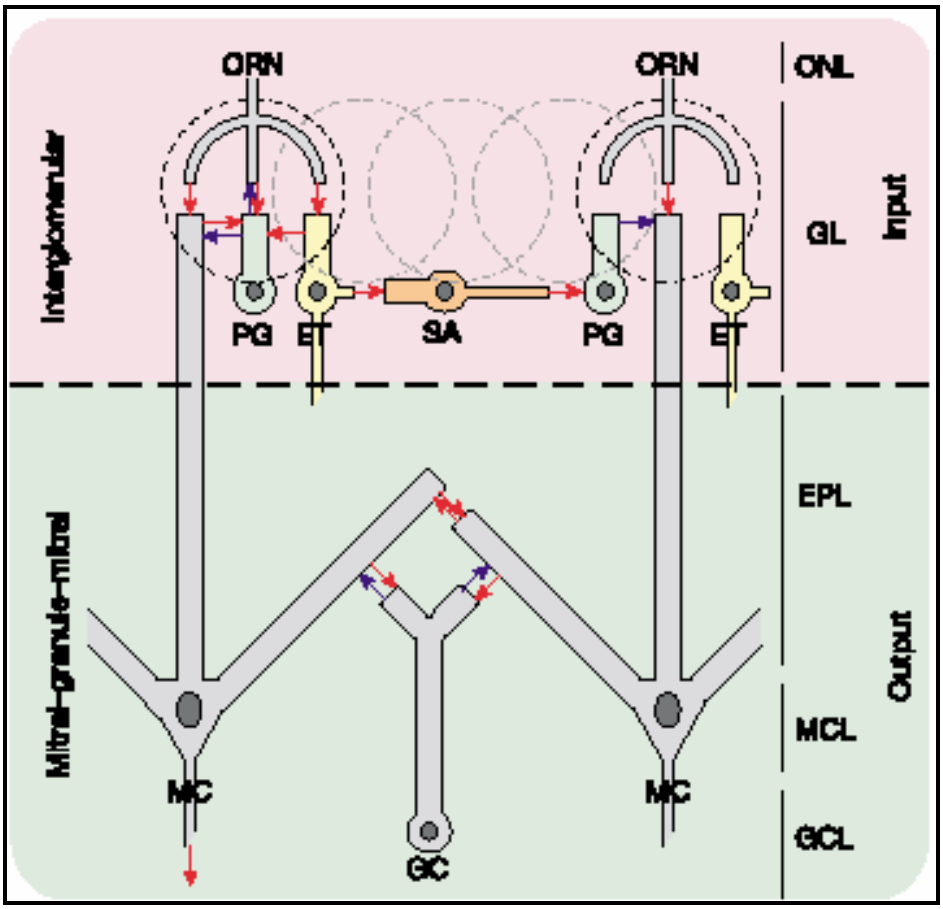

Fig I.3 Figure showing two serial levels of MOB inhibitory circuits. The upper half shows the on center-off surround inhibitory network of the short axon cells. The lower half shows the mitral cell- granule cell network. ORN, olfactory receptor neuron; ONL, olfactory nerve layer; PG, periglomerular cell; ET, external tufted cell; SA, Short axon cell; GL, Glomeruli; MC, mirtal cell; GC Granule cell; EPL, external plexiform layer; MCL, Mitral cell layer; GCL, Granule cell layer. Red arrows show excitation, blue arrows inhibition. From: Nature. 2003 Dec 11; 426 (6967):623-9.

2. Odor induced activity; spatial and temporal patterns

2.1 An overview of methods to study odor induced activity patterns

Many methods are available to study the activity patterns of OSNs glomeruli, bulbar neurons and the higher brain regions upon odorant stimulation (Korsching, 2001). There are non-invasive methods like Positron emission tomography (PET), Magnetoencephalography (MEG) and functional magnetic resonance imaging (fMRI). These methods have very low spatial resolution to allow imaging of individual neurons and even glomeruli. MEG measures tiny magnetic fields generated by current flows in active neurons .It has low spatial resolution, but a high temporal resolution. PET measures blood flow changes regionally via positron emission from injected tracers. BOLD fMRI measures depletion of blood oxygenation level via the influence of paramagnetic deoxyhemoglobin on proton magnetic resonance. In general, these methods have proved very useful in human studies and in identifying brain regions involved in odor coding. Calcium and voltage sensitive dyes loaded into the neurons at the nasal epithelium have been used to study bulbar activity using novel powerful methods of optical imaging. Optical imaging has also been used to monitor intrinsic signals (which arise from blood flow changes, oxygenation state of hemoglobin etc) on the bulbar surface upon odor stimulation. These methods have good temporal and spatial resolution and have been used extensively in olfaction. Odorant stimulated uptake of radiolabelled 7-deoxyglucose revealed by autoradiography is also useful to monitor odor induced activity. This method and monitoring *C-fos* expression (see below) are one shot type, in that they allow only one imaging per animal.

2.2 Optical imaging in different species; spatial patterns

Optical imaging merits special mention in this study because it will be used here and it allows repeated imaging with glomerular resolution. Optical imaging studies have been

done in honeybee, Drosophila, zebrafish, turtle and rodents. In mouse, in addition to calcium imaging, imaging of intrinsic signals has also been possible.

Honeybee and Drosophila have that advantage that individual glomeruli have stereotyped positions and can be identified based on this criterion alone. In honeybee calcium imaging experiments have been used to reveal coding at the glomerular level [Galizia, 2000 #21(Galizia and Menzel, 2001)]. In the more genetically tractable Drosophila, a recent publication outlines the construction of a genetically encoded activity marker (cameleon) for the olfactory system (Fiala et al., 2002). Studies in both species support the combinatorial mechanism of odor coding. The one glomerulus –one receptor rule has been shown to be true in Drosophila (Vosshall et al., 2000). The coding has also been shown to be chemotopic, that is, related odorants map to closer regions in the bulb. Combinatorial and chemotopic coding of odorants have been shown using calcium imaging for zebrafish (Friedrich and Korsching, 1997; Friedrich and Korsching, 1998). Here also, responses to a particular odorant is restricted to a small proportion of the bulbar region and related odorants elicit similar response patterns. Accordingly, subregions can be recognized in the zebrafish olfactory bulb for amino acids, bile acids and nucleotides. A series of amino acids activated glomeruli in the lateral region while bile acids activated the medial region. In a turtle species that senses airborne odorants, experiments showed that a large number of glomeruli were activated by particular odorants the identitiy of which changed little with concentration. The coding method is combinatorial. In mouse it was found that odorants evoked activity in subregions of the dorsal olfactory bulb based on their structural features, the most important of which is the functional group attached to the carbon chain (Uchida et al., 2000). Aldehydes and acids activated the antero-medial region while alcohols activated a lateral region. Within these subregions, the patterns differed slightly based on differences in carbon chain length and side chains. Thus the mechanism is combinatorial and chemotopic (Wachowiak and Cohen, 2001).

2.3 Temporal activity patterns in olfactory bulb and antennal lobe

In addition to the spatial activity patterns described above, which are a property of mainly the olfactory receptor neurons, there is temporal activity patterning which arise as a result of the network interactions in the olfactory bulb. These have been studied by electrophysiological methods. To begin with, the temporal activity pattern of projection neurons is made up of successive excitatory and inhibitory phases, lasting hundreds of milliseconds. It means that the ensemble of active mitral cells (projection neurons in insects) change over the course of a stimulus. Thus, information is presented not only in the instantaneous firing pattern ('odor image'), but also in the sequence of patterns ('odor movie').

In addition, as the activity patterns evolve with time, the relation between them changes. In zebra fish, at response onset, closely related molecules evoke similar activity patterns meaning that the follow initially the afferent input. But with continued stimulation, these patterns became decorrelated, and so easier to discriminate (Friedrich and Laurent, 2001). It has been proposed that these differentially processed neural responses could be used for complimentary tasks; the initial similar response could signify that the odorant(s) belong to the same class (e.g., neutral side chain amino acids) and the later decorrelated activity discriminate between them (e.g,tyrosine vs tryptophan). It is found that with time the response of projection neurons becomes synchronized with time and pharmacological abolition of synchrony affects odor discrimination (Laurent et al., 2001). Also, odor responses change over longer time scale because of a short-term plasticity mechanism. In locusts, at the onset of response to an unfamiliar odor, a projection neuron fires intense bursts of action potentials, but lacks fine temporal precision. The spikes of simultaneously recorded PNs are un-correlated and a LFP (Local Field Potential) oscillation is absent. Over prolonged or repetitive stimulation, firing rates decrease, PNs synchronise and LFP oscillations emerge (Friedrich and Stopfer, 2001). Such a phenomenon is also found in Zebra fish.

3. Experimental strategy

Optical imaging with externally applied dyes like calcium green-dextran can be done only in the first order receptor neurons (Fried et al., 2002). The mitral cells and the bulbar neurons and the neurons higher up in the odor sensing machinery are anatomically inaccessible to labeling with dyes. Further more dye labeling is invasive and may result in the labeling of neuronal and non-neuron cells indiscriminately. Dyes are also cleared from the neurons, making it impossible to carry out chronic imaging Dye labeling by injection works with at the most a few neurons. Advances in genetic modification of animals and identification of cell specific promoters have made it possible to label these cells genetically. Also, many variants of GFP are available, which makes calcium sensing, pH sensing and voltage sensing possible in living animals. These methods alter the physiology of the cell only slightly and can be used in real-time. They work in millisecond range within which odor recognition takes place. Monitoring the expression of the immediate early gene *C-fos* at the level of mRNA or protein is another popular method of activity mapping. Coupling of reporter genes to *C-fos* regulatory elements also allows monitoring *C-fos* expression.

My work exploits calcium sensing and the properties of *C-fos* to examine odorant dependent activity in specific cell types. The aim is to construct two different mouse lines, one in which the calcium sensing protein ratiometric pericam is expressed cell type specifically and another in which the fluorescent protein d2EYFP is expressed under the control of *C-fos* regulatory elements. Ratiometric pericam belongs to a new generation of GFP derived calcium-sensors that have been successful in cell cultures. It will be expressed under a ubiquitous promoter with a floxed transcriptional stop cassette in between d2EYFP, a short half-life version of EYFP is be hooked to *C-fos* regulatory element with the stop cassette in between. Crossing to a cre recombinase-expressing line can activate both the reporters. For monitoring the activity of mitral cells, the Tbet cre

line is constructed in the lab by a colleague. I report here the generation and analysis of the ratiometric-pericam strain and generation of the *fos*-d2EYFP strain.

II MATERIALS AND METHODS

1. Biological material

1.1 Experimental Animals

Mice of the strains C57/BL6 and DBA/BL/6 hybrids were used in the studies reported here. They were either obtained from the mouse breeding facility at the Center for Mouse Genetics, Institute for Genetics, or purchased from Charles River laboratories. The animals were housed in the same facility with 12 hours of light and 12 hours of darkness and dry food pellets and water ad libidum.

1.2 Eukaryotic cell line

Human embryonic kidney (HEK) 293 cells were used in this study

1.3 Bacterial strains

For routine amplification of plasmids, the following bacterial strains were used:

***Epicurian coli* XLI Blue MRF'** Stratagene (Heidelberg)

Genotype: (*mcr*A)182, (*mcr*CB-hsdSMR-mrr)173, *end*A1, *sup*F44, *thi*-1, *RecA*1, *gyr*A96, *rel*A1, *lac* [F', proAB,lacI^{q}Z, M15,Tn10(tet^{r})]

DH5α: RZPD (Berlin)

Genotype: *sup*E44, Δ(lac, ZYA-*arg*F), U169, (Φ80, *lac*ZDM15), *hsd*R17 (r_k^- m_k^-), *RecA*1, *end*A1, *gyr*A96,(Nal^r), *th*iI, *rel*A1

For PCR based BAC modification, the following strain was used:

DY380 (Neil Coupland, Maryland)

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15Δ*lac*X74 *deo*R *RecA*I *end*AI *ara*D139 Δ(*ara,leu)*7649 *gal*U *gal*K *rsp*L *nup*G [λ*cl*857 (*cro-bio*A) <>*tet*]

2. Chemicals and supplies

All chemicals used in this study were from Ambion (Austin USA), Amersham Pharmacia biotech (Freiburg), Applichem (Darmstadt), JT Baker supplied by Fischer scientific (Schwertze), Biozym (Hessisch Oldendorf), Calbiochem (Darmstadtt), Difco (Detroit, USA), Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelburg), or Sigma (Diesenhofen) unless stated otherwise in the text.

2.1 Enzymes

Restrictionn enzymes were either from New England Biolabs (Schwalbach Taunus) or Amersham pharmacia Biotech (Freiburg). T4 DNA polymerase, Taq DNA polymerase, Pfu High fidelity polymerase, Klenow enzyme, etc were purchased from Roche

Biochemicals (Mannheim).Shrimp alkaline phosphatase (SAP) was from USB (Cleveland, OH, USA). Rnase A and proteinase K were purchased from Sigma.

2.2 Nucleotides

Nucleotides for PCR were from Sigma. Radio-nucleotides (α -^{32}P)dCTP (3000Ci/mmole on the day of delivery)was purchased from Amersham Pharmacia Biotech.

2.3 Membranes and Filters

Nylon, nitrocellulose and PVDF membranes Hybond- N ™ and Hybond –P were from Amersham Pharmacia Biotech. GB 003 Gel-Blotting paper and sterile filters were purchased from Schleicher and Schuell supplied by Fischer scientific.

2.4 Films

X-Ray films XLS-1, X-OMAT™AR, and X-OMAT™LS in various sizes were from Kodak (Stuttgart). X-Ray film cassettes IEL6040C with intensifier screens were from Rigo (Augsberg).

2.5 Plastic ware

All disposable plastic ware like 15 ml and 50 ml Falcon tubes, 6, 24, 48 and 96 well plates, and petri-dishes, in various sizes were from TPP or Castor, both purchased through Fisher Scientific.96 well plates Polyfiltronics for colony PCR were from Whatman (supplied by Fisher scientific). 0.2 ml PCR tubes and sterile pipette tips were from MβP supplied by Fisher scientific. Non sterile pipette tips were supplied by LaFontaine (Forst/Bruchsal) and Labormedic(Bonn).

3. Preparation of solutions and reagents

Solutions were prepared with water from seralpur machines. When necessary, they were autoclaved for 20 minutes at 121 bar. When sterilization is desired but heat sterilization is not possible, they were filter sterilized (0.22.0.45 microns pore diameter). Glass-ware was baked at 180˚C for 2 hours for sterilization.

Most of the standard solutions like EDTA, Tris, TAE, TBE, TE, PBS, TBS, SDS, SSC, Sodium acetate, and culture media like LB and SOC were prepared according to Sambrook *et al* (1989). All solutions used are named in text.

4. Plasmids and vectors

Table III. I: List of cloning vectors

Name	Supplier	Properties
pBluescript II KS (+)	Stratagene	2.96 kb,B/W, amp^r
pGem-T	Promega	3 kb, B/W, T-Vector, amp^r
pGem5Z (+)	Promega	3 kb, B/W, amp^r

B/W: Blue/ White selection with IPTG and X-gal possible

Table III.2: List of Expression vectors

Name	Supplier	Properties
pEYFP1	Clontech	4.2 kb, promoterless, kan^r
pd2EYFP- NI	Clontech	4.8kb, CMV promoter, kan^r
Ratiometric Pericam pcDNA3	Miyawaki	6.6 kb CMV promoter, amp^r, kan^{r,}

EYFP1: encodes a enhanced yellow fluorescent variant of *Aequorea victoria* GFP.

d2EYFP: Encodes a short half-life variant of EYFP

Ratiometric pericam: A Calcium sensing variant of EYFP

Table III.3: List of Genomic DNA clones

Name	Supplier	Properties
ICRFP703D0529Q3	RZPD	Mouse *C-fos* locus in pAd10SacBII kan^r
RPCIB731G07234	RZPD	Mouse *C-fos* locus in pBACe3.6 Chloramphenicol resistant
RPCIB731J15158	RZPD	-Do-
RPCIB731M24459	RZPD	-Do-

Table III.4: List of Plasmids from external sources

Plasmid name	Supplier	Description
pSPfos 65	Ralf Heumann	1.3 kb mouse v-fos fragment in pSP65 vector
pBS302	Brian Sauer	1.5 kb floxed stop cassette
pSvRecA	Nathaniel Heintz	Vector for recombineering
PCAGGS	Jun-Ichi Miyazaki	CAG promoter and Rabbit βGlobin PolyA signal
pL451	Neil G Coupland	Kan^r gene with PGK promoter
GtxBRPh	Vincent Mauro	Gtx 5X IRES between renilla and photinus luciferases
Ratiometric-pericam pcDNA3	A Miyawaki	1.2 kb ratiometric-pericam in pcDNA3

5. Libraries

The P1 and BAC genomic libraries of mouse were provided by Human Primate Resource Center (RZPD), Berlin. The P1 library was created by Fiona Francis in the pAd10SacBII vector and electroporated into *E.coli* NS3145 strain. The BAC library was created by Minako Tateno et al with DNA isolated from brain and kidney and cloned in pBACe3.6 vector. The bacterial strain used was *E.coli* DH10B.

6. Primers

Oligonucleotide primers were by the suppliers Invitrogen or Sigma. The scale of synthesis was 50 nano moles and they were delivered lyophilized and dried. On delivery, they were reconstituted to a concentration of 100 µM with sterile distilled water. Working dilutions of 10µM were made for routine use. Both were stored at -20˚C.
Primers were made for different purposes like subcloning, screening, genotyping, colony PCR, reconstitution of IRES, introduction of restriction sites, Red- Gam recombination etc. All primers used in this study are listed below.

Table III.5: List of oligonucleotide primers

Primer name	**Template**	**Purpose**	**Sequence 5'-3'**
Fos-f	BAC	Screening for *C-fos*	Aaaaactggagtttattttggcag
Fos-r	BAC	Screening for *C-fos*	Tttttcattcattcaaattcactttcca
IRES2-5	Plasmid	Subcloning IRES	Tccccccgggggagcccctctccctcccc
IRES2-3	Plasmid	Subcloning IRES	Ggatcctgtggccatattatca
Ir-r	Plasmid	Subcloning	Tcgagtcgactctagagtcacggccg
Ir-f	Plasmid	Subcloning	Gctaggtcacctgagtcgacggtaccgcggg

GIR-S	None	Construction of Gtx 10X IRES	Ccggcgggtttctgacatccggcgggtttctgacatccggcgggtttctgacatccggcgggtttctgacatccggcgggt
GIR-A	None	Construction of Gtx 10X IRES	Aattcacccgccggatgtcagaaacccgccggatgtcagaaacccgccggatgtcagaaacccgccggatgtcagaaacccgccgg
Sto-f	Plasmid	Subcloning	Atgcatctagaataacttcgtatagcatacat
Sto-r	Plasmid	Subcloning	Atgcatctagaataacttcgtataatgtatgc
Giy-r	Plasmid	Colony PCR	Agtctggtcaccctagtggatccccccggc
CAG-F	Plasmid	Subcloning	Atggcggccgcacattgattattgactag
CAG-R	Plasmid	Subcloning	Atggcggccgccagctgctttaataagat
Fp 1 fin	BAC	BAC recombination; subcloning arm A	Atacgtcgacccaaagagtcgctaactag
Fos rev fin	BAC	BAC recombination; Subcloning arm A	Atccgattggactcgagtcacagatctgcgcaa Aagtcct
Fosfp2	BAC	BAC recombination; subcloning arm B	Ctcgagtccaatcggatgggaccttacctgt
Fos rp2	BAC	BAC recombination; Subcloning arm B	Atcgtcgaccctcagggtagaaaaaa
Fosfp1	BAC	Confirming fidelity of BAC modification	Atacgtcgacggcctgcctgcattcttctct
BacF	Plasmid	Red–Gam recombination	Gcgataagttcgacgccaacgatccgatcctgaa agatcagacgcaagaatgggttccgcaagctctag tcg
BacR	Plasmid	Red-Gam recombination	Gcaatgtgtcgctgtcgacggtgaccctatagtcg agggacctaataacttcggcctttcgacctgcagcc t Gcagcctgttg
Bace3.6F	BAC	Confirming fidelity of BAC modification	Gctgcatccgatgcaagtg

G91	Genomic	Genotyping cre	Atgcggtgggctctatggcttctg
G92	Genomic	Genotyping cre	Tgcacacctccctctgcatgcacg
Rmpc-1	Genomic	Genotyping pericam	Cttccagtccgccctgagcaaagac
Rmpc-2	Genomic	Genotyping pericam	Gaagcacttcaggccgtagccgaag
Stop re	Genomic	Genotyping stop	Aagtccttggggtcttctacctttc
Stop fd	Genomic	Genotyping stop	Tcctcttaagtgggaggaacataa

7. Antibodies

The following antibodies were used in this study:

Primary antibodies:

Rabbit anti-GFP polyclonal. antibody from Torrey pines biolabs.

Mouse anti –Calmodulin monoclonal antibody from Zymed.

Secondary antibodies:

Goat anti-rabbit $F(ab')_2$ HRP conjugated, from Dianova.

Goat anti-mouse HRP conjugated, from Bio-rad.

8. Dyes, substrates, Embedding media

Horse radish peroxidase substrate

Diaminobenzidine (DAB) (Vector) Brown-black precipitate.

Embedding media

Mowiol (Polyscience Inc): Embedding medium for chromogenic and flourescent substrates.

DABCO (Sigma): Bleaching retardant (2.5% in Mowiol).

Vectashield (Vector): Mounting medium for fluorescent substrates, with DAPI.

Dyes and counter-stains

DAPI (Sigma): blue fluorescent nuclear counter-stain; 5mg/ml in H_2O (stock); working dilution 1:1000.

9. Equipment

Balances	Sartorius Laboratory, Sartorius, Heidelberg
	Sartorius Universal, Sartorius
	Sartorius Handy, Sartorius
Centrifuges	Biofuge 13, Heraeus Instruments, Hanau
	Eppendorf Centrifuge 5415D, Eppendorf, Hamburg
	Eppendorf Centrifuge 5417R, Eppendorf
	Sorvall R5-BB, Dupont Instruments, Bad Homburg
	Sigma 4K 10 B, Braun, Melsungen
CO_2 incubator	Kendro/Haereus, Hanau
Computer	G3, G4, Macintosh, Apple
Cryostat	Cryostat CM 1900, Leica
Developing machine	Curix 60 Agfa
Electroblotter	Semi Dry electroblotter ltf labortechnik Bodensee
Electrphoretic equipment	Mini-Sub®Cell GT, Biorad
	Sub®Cell GT, Biorad
	Power Pac 300 Biorad
	CHEF-DR® III Pulsed field Gel electrophoresis systems
Electrporator	Gene pulser™, Biorad
	Pulse controller (Biorad, Munich)
	Capacitance extender (Biorad, Munich)
Heating Block	Thermomixer comfort 1.5 ml, Eppendorf
	Hybaid Intelligent heating block, Biometra
	Dri.Block™ DB-3 Techne, Cambridge, UK
Hybridization oven	Incubator 1000, Heidolph
	Techne, Cambridge, UK
Microscopes and accessories	Axiophot (Photomicroscope Pol), Zeiss
	Light source: Atto Arc®2 HBO 100W Zeiss

	Objectives: Plan-Neofluar:10X NA0.3,20X NA 0.5
	Filter: 485 BP-436/10FT 510 BP 515-565
	RT slider SPOT Camera, Diagnostic Instruments
	Uniblitz® VMM-D1 Shutter driver, Vincent associates
	Metamorph 4.0, Universal Imaging Corporation
MilliQ water	Seralpur DELTA, NSF Seral
pH meter	pH Meter 766 Calimatic,Knick
Photometer	UNICAM 8625 UV/VIS Spectrophotometer
Printer	Laser writer,Epson EPL-6200, Epson stylus colour 760
Scanner	Agfa SnapScan1236
Shaker	IKA-VIBRAX-VXR, Janke and Kunkel
Sterile Hood	Heraus Lamin Air LFM2448 S, Heraus, Hanau
Transilluminator	UV-Transilluminator, Stratagene
	Gel Doc System, Biorad
	Quantity One 4.2.1, Biorad
	Herolab UVT 2020 The Imager™
	High performance CCD-Imager system, Biometra
Thermocycler	Gene Amp PCR system 2400, Perkin Elmer
	T gradient Biometra
UV crosslinker	UV stratalinker 1800, Stratagene
Vortex machine	Vortex Genie 2™, Bender & Hobein, AG,Melsungen
Waterbath	Thermomix, B, Braun

10. Molecular biological methods

Standard molecular biological methods like plasmid DNA preparations, restriction digestions, agarose gel electrophoresis, 5' and 3' end modifications, dephosphorylation etc were performed as described in Molecular Cloning (Sambrook *et al* 1989). When using kits, manufacturer's instructions were followed.

10.1 Isolation, purification, and quantification of DNA

10.1.1 Isolation of plasmid DNA

Plasmid DNA preparation was done either with commercially available kits or lab made reagents. The preparations were either small scale (called miniprep in the lab parlance), employed for clone identification, sub-cloning, sequencing etc or large scale (midiprep) for microinjection into mouse pro-nuclei. All these methods were modified from the alkaline lysis method of Birnboim and Doly (1979).

For minipreps the desired colony from transformation or glycerol stock was inoculated into Luria-Bertani broth (10g tryptone,5g yeast extract, and 10g NaCl per liter, pH 7.0) containing the appropriate antibiotics (ampicillin: 100µg/ml, tetracycline:50µg/ml, chloramphenicol:20µg/ml and kanamycin:50µg/ml) in a volume of 4 ml and grown in an orbital shaker at 37˚C overnight.

The bacteria were pelleted by centrifugation and subjected to alkaline lysis. In cases were kits were used, the supernatant from alkaline lysis was loaded onto anion exchange columns provided by thee manufacturer. RNA and protein impurities were removed by a medium salt wash and DNA was eluted in a low salt buffer. In case of hand made minipreps, the supernatant from alkaline lysis was subjected to isopropanol precipitation and ethanol wash. The yield from a miniprep is typically 10-20 µg,

For midipreps, which typically yield about 200 µg plasmid DNA, a kit provided by quiagen was used. The culture volume was 100 ml. The bacterial pellet was subjected to alkaline lysis and the supernatant was loaded onto anion exchange column that was processed as per the manufacturer's instruction.

10.1.2 Preparation of BAC DNA

BAC DNA was prepared in small amounts exactly as per the miniprep protocol, with isopropanol precipitation. Yields were about 5 µg, which is enough for one restriction enzyme digestion. For large-scale preparation of BAC DNA for microinjection of mouse pronuclei, BAC DNA was isolated from a culture volume of 400-ml using the BAC maxi-kit from promega, which combines alkaline lysis with column chromatography. Yield was typically in the range of 100 -150µgs.

10.1.3 Preparation of genomic DNA from mouse tail biopsies

A piece of tail about 1.5 cm in length was cut from the mouse and incubated in lysis buffer (100mM tris HCl pH8.5, 5mM EDTA, 0.2% SDS and 200mM NaCl) with about 100µg proteinase K at 55˚C overnight. The digested sample was centrifuged at full speed in a microcentrifuge for 10 minutes to pellet hair and the supernatant was subjected to isopropanol precipitation followed by 70% ethanol wash. The pellet was dissolved in 200µl TE by incubating at 60˚C for three hours. This yielded DNA that was pure enough to be digested with EcoRV and EcoR1, the enzymes used in this study.

10.1.4 Phenol chloroform extraction of DNA

DNA solutions with protein contamination were cleaned up by phenol-chloroform-isoamylalcohol (25:24:1) extraction. DNA solution volume was adjusted to at-least 200µl using distilled water and an equal volume of PCI was added to this solution and vortexed. It was then centrifuged for 3 minutes and aqueous phase was transferred to another tube and extracted with equal volume of chloroform. The aqueous phase was then ethanol precipitated as described below.

10.1.5 Ethanol precipitation of DNA

To remove salts from DNA, ethanol precipitation was employed. Sodium acetate concentration in the DNA solution was adjusted to 0.3 M and two to three volumes of absolute ethanol was added. Precipitation was allowed at -20˚C for 20 minutes. It was then centrifuged at maximum speed in a refrigerated micro-centrifuge at 4˚C. The pellet was washed with 70% ethanol, air-dried and re-suspended in the appropriate solution.

10.1.6 Quantification of DNA samples

DNA was quantified by UV spectrophotometry. An optical density of 1 corresponds to a DNA concentration of 50µg/ml. Alternatively, a rough estimate of DNA concentration was obtained by electrophoresis of DNA of unknown concentration alongside standards of known concentration. This method was especially useful in cases where the amount of DNA in hand was low.

10.2 Agarose gel electrophoresis of DNA

10.2.1Routine agarose gel electrophoresis of DNA

For routine gel electrophoresis of DNA 1% agarose gels containing 0.5µg/ml ethidium bromide in 1x TAE buffer were run at 5-10 V/cm. The DNA staining persisted in these conditions although ethidium bromide runs in a direction opposite to that in which DNA runs.

10.2.2 Pulsed Field Gel Elecctrophoresis

For separating BAC DNA or BAC DNA digests (fragment sizes from 250 Kb to 10 Kb) PFGE was frequently employed. The system available was CHEF-DR III (Clamped Homogenous Electric Fields - Dynamic Regulation) Pulsed Field Gel Electrophoresis

apparatus from BioRad. It uses an array of 24 electrodes in groups of four which are autonomously controlled and clamped or held to intermediate potentials upon sensing variations in local buffer conductivity, which results in straight lanes. BAC DNA was loaded into pockets using loading buffer for normal agarose gel electrophoresis in 1% PFGE grade agarose in 0.5x TBE and run for 13 hours with switch conditions ramped from 0.1 to 6 seconds in a field of 4.8 V/cm at an angle of 120˚.

10.3 Enzymatic manipulation of DNA

10.3.1Restriction endonuclease digestion of plasmid DNA

Plasmid DNA was frequently digested for sub-cloning, restriction mapping and so on. Routine digestions were carried out in a volume of 20 µl on about 200-500ng of plasmid DNA. 3-5 units of restriction enzymes were used for this amount of DNA and digestion was carried out with the prescribed buffer at 1x concentration at the appropriate temperature (usually 37˚C, 30˚C or 25˚C) for about 1-3 hours. BAC DNA was digested in the presence of 2.5mM spermidine along with the prescribed buffer.

10.3.2Enzymatic Digestion of mouse genomic DNA for southern blotting.

Digestion of mouse genomic DNA for southern blotting was done by a special protocol. About 10µg of genomic DNA isolated from tail biopsies was digested in a volume of 200µl overnight using about eighty units of EcoRV or EcoR1 at 37˚C. The digest was 'cleaned' by ethanol precipitation and the pellet was suspended in 30µl of TE and loaded onto agarose gels

10.3.3 Dephosphorylation of 5' terminus of restriction digested DNA

When desired the 5' termini of vector DNA fragments were treated with shrimp alkaline phosphatase to prevent vector re-ligation. Briefly, about 100ng of digested vector was treated with 1 unit of shrimp alkaline phosphatase in the manufacturer's buffer as directed. The enzyme was then heat inactivated at 65˚C and the mix was directly used for ligation. Alternatively, it was heat inactivated and purified by drop dialysis for use in ligation.

10.3.4 Blunting 5' and 3' overhangs

For filling of 5' overhang in digested DNA fragments, Klenow polymerase fragment was used. The dNTP concentration was adjusted to 33μM and 1 unit of enzyme was added. The reaction mix was incubated at 37˚C for 30 minutes, heat inactivated and purified.
For filling 3' protruding ends, dNTPs were added to a final concentration of 2mM and incubated at 12˚C for 15 minutes with 1 unit of T4 DNA polymerase per microgram of DNA. The enzyme was heat inactivated at 70˚C and the DNA was purified.

10.3.5 Ligation of vector to insert

Ligation reactions were used to clone insert DNA into the appropriate vector. For this purpose, purified dephosphorylated vector DNA was mixed with insert DNA in a 1:3 molar ratio (experimentally varied for different ligation reactions) and incubated at 16˚C overnight with 1 unit of phage T4 DNA ligase in the manufacturer's buffer. Some times, 5% Poly ethylene glycol (PEG 8000) was found to promote ligations especially if it involved blunt DNA ends. PCR fragments were cloned into the P-GEM T-Vector using the P-GEM T-Vector ligation kit as per the manufacturer's instruction.

10.3.6 Isolation of DNA from agarose gels

The desired band of DNA was cut out from the agarose gel and processed according to the instructions for the kit used (generally QIAquick gel extraction kit or High pure PCR product purification kit) Both kits employ steps wherein the gel is digested in a solution of chaotropic salts and the resulting DNA solution is passed through a column with silica membrane to bind DNA. The contaminants are washed away with an ethanolic salt solution and the DNA is eluted with low salt buffer.

10.4 Electroporation of DNA into bacterial cells

10.4.1 Preparation of electrocompetent E.coli.

E.coli of the strain XL1Blue or JM 109 was used for routine electroporation. Electrocompetent cells were prepared according to the method of Dower (1988) by washing a log phase bacterial culture pellet with ice-cold sterile distilled water several times and finally re-suspending in 10% glycerol. The cells were then aliquoted and stored at -80˚C.

10.4.2 Electroporation of DNA into bacterial cells

For electroporation, the electrocompetent cells were thawed on ice, mixed with 2-3 µl of ligation mix and incubated for 1minutes. Electroporation was performed in 1mm cuvettes in the gene pulser (Biorad) at E=1.8KV/cm, C=25µF and R= 200 ohms. 1ml of prewarmed LB medium was added to the bacteria and they were allowed to recover and express the selection marker by incubating them in a shaker at 37˚C for 1hr. An aliquot was then plated on LB agar plates with the appropriate antibiotics. X-gal and IPTG were added to the plates when blue –white selection was desired.

10.5 Identification of recombinant clones

Recombinant clones were identified by several methods. Blue white selection was used when possible. In other cases, a colony PCR was performed with primers directed against or flanking the insert. Basically a small portion of the colony was transferred to a PCR mix with the appropriate primers and subjected to PCR after an extended initial denaturation period. PCR protocol is as described later. The product was run on an agarose gel and the DNA was visualized by ethidium bromide staining. Colony hybridization was also used on some occasions to identify positive clones (for protocol, see section 10.10).

10.5.1 Preparation of glycerol stocks.

Glycerol stocks were prepared from positive clones by adding 150 µl of sterile glycerol to 850µl of bacterial culture, vortexing to ensure even dispersion of glycerol and freezing in liquid nitrogen. The cultures were kept at -80˚C for long term storage.

10.6 Polymerase chain reaction

Several applications in molecular biology like sub-cloning, identification of positive clones, determination of insert size, addition of restriction sites to DNA termini via primer design etc require PCR. A PCR reaction mix contains 1X PCR buffer, 0.2mM each dNTPs, 1.5mM MgCl2, 1pmol/µl primer (each) and IU Taq polymerase (home made) per 20µl reaction mix.. When it was desired to avoid PCR induced mutations, a proofreading polymerase like Pfu was used, as directed by the manufacturer. Generally the reaction was allowed to run for 35 cycles.

PCR primers were designed using the primer 3 or DNASIS program and annealing temperatures were always kept optimal. It was taken care that the sequences included no hairpins and no primer dimers could be formed. Whenever possible, PCR was simulated using the Amplify 1.2 program.

10.7 DNA sequencing and sequence analysis

10.7.1 Routine DNA sequencing

DNA sequencing was done by chain termination in a PCR machine. The reaction was performed in a volume of 10µl containing 2µl of terminator-buffer-enzyme premix (big dye v 2.3, ABI prism), 500ng of purified plasmid DNA and five pmol primer. The reaction conditions were

96˚C for 2 minutes

(96˚C for 30 s, 50˚C for 15 s, 60˚C for 4 min) 25 cycles.

The samples were ethanol precipitated and dried thoroughly. The dried samples could be stored in -20˚Cin the dark until they were electrophoresed on a sequencing gel. Electrophoresis was carried out by Rita Lange in an ABI prism™ 377 sequencer (applied bio-systems) in the core facility of the Institute for Genetics.

10.7.2 BAC end sequencing

To position a BAC with respect to the mouse genome sequence, its ends were sequenced with the T7 and SP6 promoter primers that anneal to the backbone of the BAC in regions that flank the insert. The reaction mix contained 1µg BAC DNA, 8µl big-dye mix, 5 pmol T7 or SP6 primer, and the appropriate buffer in a reaction volume of 20 µl. The cycle parameters were as follows

94˚C 5 min

(94˚C 30 s, 50˚C 15 s, 60˚C 4 min) 50 cycles.

The reaction product was processed as before and run on a sequencing gel at the core facility.

10.7.3 Sequence analysis

Sequence comparisons and other analyses were performed through the online resources available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) which includes several databases and search programs. Mainly BLAST 2.0 (Gapped BLAST and graphical viewer) with the advanced BLAST option was used. Also used was the DNASIS program and Vector NTI.

10.8 Radioactive labeling of DNA

Radioactively labeled probes for membrane hybridization of BAC or genomic DNA blots were prepared with the Prime -It II Random priming kit from Stratagene. 25-50 ng of double stranded DNA was denatured by boiling in a water bath together with 94.5 OD of an oligo $(N)_9$ primer mix for 10 minutes. It was allowed to cool down at room temperature to allow annealing of oligos to the DNA template. A 5X primer buffer was added which contained dATP, dTTP, and dGTP at 0.1 mM each, followed by (α -^{32}P) dCTP and 5U of Exo (-) Klenow. The reaction was incubated at 37˚C for 1 hour and purified using NICK™ column (Sepahdex G-50 column) from Amersham Pharmacia Biotech. According to the manufacturer's protocol. The amount of radioactively labeled probe was quantified by scintillation counting.

10.9 Southern transfer and membrane hybridization

10.9.1 Southern blotting

Southern blotting is a technique where by DNA in an agarose gel is transferred to a nitrocellulose membrane to allow membrane hybridization. Briefly, DNA is subjected to gel electrophoresis. The gel is then treated with 0.25 M HCl for 5 minutes to depurinate the DNA. It is then treated twice for 15 min each with denaturing solution (0.5M NaOH, 1.5 M NaCl) to break DNA strands at the sites of depurination and again twice for 15 minutes each with 1M ammonium acetate. It is then put on an appropriately sized nitrocellulose membrane for a downward capillary transfer in 1M ammonium acetate. The blotting was conducted overnight to ensure a complete transfer and then cross-linked to the membrane by exposure to UV light of appropriate strength in a UV crosslinker. The membrane is now ready for pre-hybridization.

10.9.2 Pre-hybridization and hybridization

Membranes that were either spotted with colonies from genomic libraries or subjected to southern transfer were hybridized to appropriate radioactively labeled probes. Briefly, they were pre-wetted in 2X SSC and put DNA side facing inwards into a hybridization tube. Pre-hybridization was done using Ultrahyb™ hybridization solution (Ambion) for 30 minutes to 1hour (longer for genomic southern blots) at 42˚C in about 10-15 ml of solution to keep the membrane uniformly wet.
Radiolabeled DNA probes were denatured by adding 3µl/100µl of 1M NaOH and heating at 60˚C for 10 minutes. Probe concentrations of about 10^6 cpm/ml were used for hybridization which was run overnight.

10.9.3 Post-hybridization washes

After hybridization, the hybridization solution containing the un-hybridized probe was discarded and the blots were washed two times for 5 minutes each in 2X SSC, 0.1%SDS

and then two times in 0.1X SSC, 0.1%SDS for 15 minutes each. The membrane was then put DNA side facing up on a filter paper, covered with saran wrap and exposed to X-ray imaging film. The films were developed in the automatic X-ray sheet developing machine (curix).

10.9.4 Stripping and re-probing of membrane

If desired, the hybridized probes were stripped from the membrane to allow re-probing with different probes. This was done as follows. The membrane was incubated in 0.4N NaOH at 45˚C foe 30 minutes. It was then transferred to a solution containing 0.1X SSC, 0.1% SDS and 0.2 M Tris pH7.5 and incubated for a further 15 minutes. It was then autographed to see if there is any residual probe bound. The membrane was now ready for re-probing.

10.10 Colony Hybridization

Colony hybridization was used to identify bacterial colonies that carried the cloned DNA. The procedure involves transferring a replica of the colonies to nylon membrane, lysing the cells, fixing the DNA and probing it with radioactively labeled probe of interest. Positive clones can be picked by aligning the developed film to the agar plate containing them. The procedure is outlined below

10.10.1 Transfer of colonies to nylon membrane

Bacterial colonies at a density of not more that 200 per plate are grown overnight to an appropriate size. The plates are then cooled to 4˚C and the nylon membrane is carefully placed over the colonies. Parts of the colonies adsorb to the membrane and the position and orientation of the membrane with respect to the plate is marked on the agar by

driving pins through the edges of the membrane into the agar asymmetrically. The membrane is peeled off from the agar surface after 30-60 seconds and treated as outlined below

10.10.2 Cell lysis and DNA fixation

DNA must be liberated from the bacteria and fixed on the membrane. For this purpose, the membrane is first placed for 3-5 minutes bacteria side up on filter paper soaked in denaturation solution (1.5M NaCl, 0.5M NaOH). It is then placed on a filter paper soaked in neutralization buffer (1.5M NaCl, 0.5M tris, pH7.5) for six minutes. It is then washed vigorously in 2X SSC and cross-linked to the membrane by exposure to UV light in a cross-linker. Hybridization and development is as outlined in section 1.9.

11. Cell culture experiments

11.1 Maintenance of cells in culture

Human embryonic kidney cells (HEK 293 cells) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) supplemented with 10% Fetal Calf Serum (FCS) (Roche Biochemicals), 100U/ml penicillin, 100µg/ml streptomycin and 2mM glutamine in a humidified 5%CO_2 incubator at 37˚C. The cells were split 1:3 every five days to maintain healthy growth.

11.2 Lipofectamine mediated transfection of cells in culture

Cells were transfected at about 50% confluence.500 ng plasmid DNA was mixed with 2µl Lipofectamine along with 250µl OPTIMEM for each well in a 24 well plate and

allowed to incubate for 15 minutes. The growth medium was aspirated and substituted with the DNA mix and incubated for 5 hours. The transfection mix was then pipeted out and fresh growth medium was added. For stimulation of *C-fos* transgenes, the transfected cells were treated with 25 nM phorbol 12-myrisitate 13-acetate (a phorbol ester). The fluorescent proteins could be observed several hours after stimulation.

12. Targeted modification of Bacterial artificial chromosomes in- vivo

12.1 Modification of BACs by *RecA* mediated recombination in *E.coli* (Heintz method)

A method for targeted modification of BACs in-vivo was developed in the laboratory of Nathaniel Heintz in 1997. This method can be used for deletion, substitution and insertion of DNA into BACs. The details of the method are outlined elsewhere in the thesis. The method involves the following steps

12.1.1 Cloning of the recombination cassette

Two PCR fragments flanking the region to be modified (the arms) are cloned from the BAC. These regions should be about 500 bp each in length, should not contain internal Sal1 or Not1 sites and Sal1 sites are introduced at the 5' and 3' ends by incorporating them at the 5' end of primers. They are linked together in the original orientation with a restriction site in between for cloning the sequences to be inserted. These sequences are then cloned in between the arms in the proper orientation to generate the recombination cassette. The cloning is done in an ordinary cloning vector called building vector.

12.1.2 Subcloning of the recombination cassette into pSV.RecA shuttle vector

pSV1.*RecA* vector is prepared by midiprep method and digested with Sal1 and de-phosphorylated. The recombination cassette is excised with Sal1 and cloned into the shuttle vector

12.1.3 Homologous recombination-step 1-identification of cointegrates

The pSV1.*RecA* vector carrying the recombination cassette is electroporated into *E.coli* cells containing the BAC to be modified. These cells are then grown overnight at 30˚C in LB plates containing tetracycline and chloramphenicol. A thick lawn of bacterial cells result. About six colonies are picked from these and suspended in I ml LB medium. I00µl of which is spread on a plate containing tetracycline and chloramphenicol. and grown at 43˚C overnight. A few big colonies are observed to be growing on a background of many satellite colonies. These are the co-integrates, i.e., cells in which the plasmid has integrated into the BAC by recombination. About twenty of these colonies are picked and inoculated into LB medium and streaked on LB plates (both containing tetracycline and chloramphenicol) and grown overnight. BAC DNA is isolated from the liquid culture and digested with an appropriate enzyme and subjected to southern blotting and membrane hybridization with a suitable probe to reveal the presence of the recombined shuttle vector.

12.1.4 Homologous recombination step 2-resolution of co-integrates

Once correctly recombined co-integrates are identified, several of these are streaked on a LB plate containing chloramphenicol alone and allowed to grow overnight at 43˚C. Sans tetracycline, they lose the plasmid sequences by a second set of recombination (which can also result in the loss of the recombination cassette if it occurs at the same homology arm as the first recombination; if it occurs in the other arm, it results in the removal of

only the plasmid sequences). To select for this event, several colonies from this plate are streaked on to plates containing fusaric acid and chloramphenicol, which are made as follows:

10 g tryptone, 1g yeast extract,1g glucose,8g NaCl,0.5ml 0.1M$ZnCl_2$, 8 ml chlor-tetracycline(6.3mg/ml) and 15 g agar are suspended in I liter tap water and autoclaved. Also autoclaved is 500ml 1M NaH2PO4. After the temperature of the autoclaved solution drops to 60˚C 72 ml of NaH2PO4 solution and 6 ml of 2mg/ml filter sterilized fusaric acid solution and 1 ml of 20mg/ml chloramphenicol solution are added and the plates poured.

These plates are incubated with the bacteria for 2-3 days to allow the growth of cells that have lost tetracycline marker (they grow slowly because of the fusaric acid). Then individual clones are picked grown in LB medium with tetracycline, and DNA extracted from them. Southern blotting after digestion with appropriate enzymes and hybridization with a suitable probe identifies the correctly recombined BAC clones. One or two clones are selected from this screen and extensive restriction mapping assesses their fidelity.

12.2 Modification of BACs by the Red-Gam system (Coupland Method)

The Red-Gam method of modifying BACs uses the (phage Gam inhibitor of RecBCD and the Red recombinase proteins Exo and Beta contained in a prophage integrated in bacterial strains like DY380 for targeted recombination. It can work with short (about 40 bp) regions of homology and can use linear DNA as substrate. Thus PCR products with appropriate selection cassettes included can be used for modification. In this work, this method is employed for replacement of a region from a BAC clone. A description of the method is given in the result section. Here, the methodology is given.

12.2.1 Electroporation of BAC into DY380 cells

DY380 cells are DH10B cells containing the red-gam prophage and are competent for BAC electroporation. Electro-competent DY380 cells are prepared as usual and electroporated with 2 (g of BAC DNA at 25(F, 1.25 KV and 100 ohms. The cells are shaken at 32˚C for 1.5 hours and an aliquot is plated on LB agar plates with chloramphenicol and grown at 32˚C overnight. A few clones are picked and checked for the fidelity of the BAC by digesting isolated DNA with restriction enzymes.

12.2.2 RED Gam induction and preparation of competent cells

The DY380 cells containing the BAC are grown in 5 ml LB medium at 32˚C overnight with chloramphenicol. 1 ml of the overnight culture is inoculated into 50 ml of fresh LB and grown at 32˚C till the OD600 value is about 0.6. RED induction is performed by transferring 10 ml of this culture into a 125 ml conical flask and incubating it in a shaking water bath set at 42˚C for 15 minutes. Immediately afterwards, the flask is transferred to an ice-bath slurry and shaken by hand to cool down the cells. The cells are then spun down at 4˚C and the pellet is washed three times with 5ml ice-cold sterile water in 1.5 ml eppendorf tubes. After the final wash, the cell pellet is suspended in 100µl ice-cold sterile distilled water for immediate use in electroporation

12.2.3 Designing PCR products for recombination

The first step is to choose a drug selection marker (Kanamycin) as the fragment that replaces the desired region in the BAC. Primers are then designed with a 5’ end of about 35-50 bases that is homologous to the region flanking the target in the BAC and 3’ end homologous to the replacement cassette. PCR is carried out with the plasmid containing the drug selection marker as template. Traces of template DNA remaining in the PCR product is removed by treating it with the restriction enzyme DpnI which cleaves at

methylated GATC sites.

12.2.4 Transformation and selection

About 100ng of the PCR product is mixed with 50µl electrocompetent cells and transferred to an electroporation cuvette. Electroporation is carried out under the following conditions: 1.8 KV, 25µF, and 200 ohms. One ml of LB medium is added to the cells and they were grown at 32˚C for one and a half hours. An aliquot form this is spread on LB Kan plates to select for the clones that has the PCR cassette integrated. The integrity of the insertion is ascertained by PCR using primers flanking the site to insertion and with in the insert. and by restriction mapping coupled with southern hybridization.

12.3 Column chromatography

Column chromatography was used to separate backbone from insert after digesting the modified BAC with NotI. Briefly, the column material (Sephacryl S 1000 superfine) was washed several times in injection buffer (10mM Tris, 1mM EDTA, 100mM NaCl, pH 7.5) and poured without creating air bubbles into a 25cm long column plugged with glass wool. It was packed under gravity and the digest mixed with bromophenol-blue was added on top of it just after the buffer reached the same level as the column. A reservoir was placed on top of the column and the DNA was eluted with the injection buffer after it entered the column. Fractions of 500µl were collected and analyzed by agarose gel electrophoresis.

13. Generation and breeding of transgenic mice

13.1 Pro-nuclear micro-injection

Pro-nuclear microinjection was done by Neil Smyth at the Center for Biochemistry of thee Medical faculty, University of Cologne. Hybrid ova from F1 DBA/C57BL6 mice were collected after super-ovulation of females of three weeks of age with 5U of Follicle Stimulating Hormone (FSH) at –2 days and 5U of LH immediately before pairing. Pro-nuclear micro-injection was carried out at E 0.5 with DNA at a concentration of 0.5-1µg/ml of injection buffer (10 mM Tris/Cl pH 7.5,
0.1mM EDTA pH 8.0, 100 mM NaCl, 30 µM Spermine, 70 µM Spermidine). They are transferred to psuedo-pregnant females mated to vasectomised males the same day. Ova are transferred 15 per side to both sides of the mouse into the oviduct.

3.2 Breeding and analysis of transgenic mice

Tail biopsies from F0 mice were taken and DNA isolated from them. It was subjected to southern blotting and membrane hybridization with suitable probes to identify mice that carried the injected DNA. Positive mice were bred to C57/BL6 mice and the progeny were analyzed in the same manner to confirm germ-line transmission. Positive F1 mice were bred again and a PCR with high annealing temperature (60-66˚C) was developed to identify positive progeny in further breeding.

14. Protein techniques

14.1 Western blotting of mouse brain extracts and immunodetection

Transgene expression in mouse was probed using western blotting of tissue extracts from brain. The procedure involves polyacrylamide gel electrophoresis, western transfer, and immunodetection. An outline is given below.

14.1.1 Protein extraction from mouse brain

The brain was dissected out from mice and the fore-brain was excised with a clean scalpel and transferred to 1.5 ml RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxcholate, 0.1% SDS) and lysed with a few strokes in a dounce homogeniser. The solution was spun for 10 minutes at maximum speed in a micro-centrifuge and the supernatant was saved and subjected to protein estimation.

14.1.2 Protein assay

The protein assay was done by bicinchoninic acid (BCA) method using a kit provided by Sigma-Aldrich. Briefly, reagent A (bicinchoninic acid solution) and reagent B (CuSO4) were mixed in a ratio of 50:1. 1 ml of this mixture was pippetted out into an eppendorf tube and 1µl of the protein solution was added to it. The mixture was incubated at 60˚C for 15 minutes and absorbency was measured at 562 nm against a blank mixture that contained no protein solution. The values were compared to a standard curve obtained by using known concentration of BSA and the protein concentration read off from the curve.

14.1.3 Poly-acrylamide gel elctrophoresis

Polyacrylamide gel electrophoresis was done as per Sambrook et al (1989) using the discontinuous buffer system. The resolving gel was cast with 13% acrylamide-bisacrylamide in tris buffer of pH 8.8 and the stacking gel with the standard 4% acrylamide-bisacrylamide in tris buffer of pH 6.8. 50-100µg of protein was loaded per well and it was run at constant current for two to three hours in tris-glycine electrophoresis buffer (pH 8.3) by which time bromophenol-blue reached the bottom of the gel.

14.1.4 Electro-blotting of protein s from gel to PVDF membrane

Proteins were transferred from the polyacrylamide gel to PVDF membrane after electrophoresis by electro-blotting in a semidry Electro-blotter. The membrane and filter papers were cut to the size of the gel and the membrane was presoaked in methanol. Then the gel, the membrane and filter papers were well soaked in transfer buffer (39 mM Glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). Three sheets of filter paper were placed on top of the anode base. The membrane and gel were carefully placed on top of the filter paper in that order and three more sheets of filter paper were kept on top of the gel. Care was taken to avoid air bubbles getting trapped in between. The cathode lid was gently placed on top of this set up and transfer was done at a constant current of 3 mA/cm2 for one and a half-hours. After transfer, the setup was disassembled and the proteins were fixed to the PVDF membrane by immersing in boiling water for 20 minutes.

14.1.5 Immunological detection of immobilized antigen

After fixing, the membrane is processed for immunological detection. The first step is to block nonspecific binding sites for the antibodies. This is done by incubating the membrane in 4% nonfat dried milk in 1X TBS (tris buffered saline, 50mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.5) overnight at 4˚C. The blocking solution is discarded and the membrane is incubated with the primary antibody solution in fresh blocking solution at the appropriate concentration for one hour. The membrane is then washed three times for ten minutes each in TBS and incubated with horseradish peroxidase coupled secondary antibody in fresh blocking solution for one hour. The membrane is again washed three times for ten minutes each in TBS and processed for chemi-luminescent detection of horseradish peroxidase activity. This was done using the lumi-light western blotting substrate (Roche). Equal volumes of solution I and solution 2 were mixed together and the membrane immersed in the mixture for up to 15 minutes. It was then sandwiched between two transparency sheets and exposed to X-ray film. The film was developed in the curix X-ray sheet developer machine.

14.1.6 Stripping and re-probing of membrane

When desired, the antibodies and the substrate were stripped from the membrane for re-probing of other antigens. This was done by incubating the membrane in freshly prepared 0.2 M NaOH three times for five minutes each. It was then thoroughly washed in a few changes of distilled water. If required the process is repeated. The membrane is exposed to X-ray sheet to see if there is residual substrate left. It can then be used for re-probing.

14.2 Immunohistochemistry

Immunohistochemistry is used to localize specific proteins or antigens in general in sectioned tissues. The protocol is described below.

14.2.1 Tissue preparation and sectioning

Mice were sacrificed by cervical dislocation. The head was cut with a pair of clean scissors and the brain and olfactory turbinates enclosed in their bony capsule were dissected out. Olfactory epithelia were further processed for decalcification follows: They were incubated in 4% PFA overnight and then transferred to 0.5M EDTA for two overnights. Then they were shifted to 30% sucrose and incubated overnight. For sectioning with a cryostat, tissues were put in TissueTek and frozen at -20˚C. Olfactory epithelia were gently squeezed with a broad tipped forceps to drive out residual air from the nasal cavity before freezing. Olfactory epithelium and the olfactory bulb were sectioned at 10µm. Sections were collected on superfrost™ slides which have a special coating and dried for three hours at 60˚C.They were used immediately or stored at -80˚C.

14.2.2 Antibody staining of fresh frozen cryostat sections

Slides were warmed to room temperature and the sections were fixed in 4% PFA for 10 min and washed three times for 10 min each in TBS (50mM Tris, 137 mM NaCl, 2.7 mM KCl, pH 7.5). Endogenous peroxidases were blocked by incubating the sections with 3% H2O2 for 20 minutes and washed two times with TBS for ten min each. The tissue was permeabilized with 0.5M Ammonium chloride and 0.25% Triton X-100 for 10 minutes and the slides were washed two times for ten min each in TBS. Blocking was done in 5% BSA and 3% Normal goat serum (NGS) for at-least one hour. The tissues were then incubated with primary antibodies in 0-8% BSA and 3% NGS at 4˚C overnight. For every experimental condition, one slide was processed without primary antibody, but subsequent treatments were same for all slides. They were then washed extensively in TBS (4X 10 min) and incubated with horseradish peroxidase coupled secondary antibodies in 0.8% BSA and 3%NGS in TBS for one hour. Detection was done using a peroxidase substrate (DAB) kit (Vector). The staining solution was prepared as follows: To 5ml of distilled water, two drops of buffer stock solution was added and

mixed well. Further 4 drops of DAB solution was added followed by two drops of hydrogen peroxide solution and two drops of nickel solution. The tissue sections were incubated with this solution at room temperature until suitable staining developed. Then the sections were washed for five minutes in water. They were then stained with DAPI for five minutes. and mounted in mowiol (Polysciences INC).

15. Optical imaging and data analysis

Images (240*320 pixels after 2*2 binning) were acquired with a cooled charge-coupled device (CCD) camera (Imago S/N 381KL 0041, TILL Photonics) at 470 nm excitation with a frame rate of 2 Hz for 20 s per stimulus, with an interstimulus period of at least 2 min. Odor was applied for 5 frames with a simple olfactometer. Odor responses were determined as $\Delta F/F$. Five consecutive prestimulus frames were averaged to determine F, and five frames at the peak of the response were averaged and lowpass filtered (Gaussian filter, SD = 14.8 μm) to obtain ΔF. Three independent trials with the same odor were averaged, normalized to the maximal response amplitude, and false color coded. The color scale was customized to achieve approximately equal representation of each major color. In one case, a sigmoidal color scale was used to obtain a larger dynamic range. The color value for $\Delta F/F = 0$ is identical in all representations. All calculations were done with the TILL VISION software.

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III.RESULTS

1. Outline of experiments

Many methods are available to monitor the active state of neurons. Such methods should be ideally highly sensitive and should not perturb the normal physiology of the neuron. One such method that has been in use for more than a decade is monitoring the levels of *C-fos* mRNA or protein upon neuronal stimulation. *C-fos* is a so-called immediate-early gene, a transcription factor that is upregulated early upon neuronal stimulation by calcium influx and voltage changes (Curran and Morgan, 1995; Hoffman et al., 1993). These studies have been done on many brain regions, including the olfactory bulb (Guthrie et al., 1993; Guthrie and Gall, 1995). Another indicator of neuronal activity is calcium itself. Calcium enters active neurons through voltage and ligand gated channels from the extracellular space and the endoplasmic reticulum (R, 2004). Axonal and dendritic termini have especially strong calcium currents and it has been monitored by injection of dyes that change intensity and/or wavelength of fluorescence upon calcium binding, like calcium green. This project aims to develop genetic methods for monitoring *C-fos* and calcium levels in cells of the olfactory system of mouse as outlined below.

Mice strains, in which the lacZ or taulacZ gene is expressed as a fusion protein with *C-fos* have been generated (Smeyne et al., 1992) (Wilson et al., 2002). In these mice, all the cells in which *fos* is induced by a stimulus express the nuclear *fos*-lacZ fusion protein that can be visualized by X-gal staining. The disadvantages with these first generation mice strains are that lacZ expression is not cell type specific and each mice can be used only once (Murphy et al., 2004). We propose to construct a mice strain in which only the desired cell types (mitral cells in this case) express the reporter and which can be used several times for imaging. For this purpose, the fluorescent marker protein d2EYFP is linked to the *C-fos* promoter, preceded by a floxed stop cassette and IRES element in that order. d2EYFP is a short half-life version of EYFP which turns over at a rate similar to that of the *C-fos* protein (half life of one and a half hour) (Li et al., 1998). Stop cassette contains a spacer sequence and a polyadenylation signal flanked by loxP sequences and

when placed before a sequence of interest, blocks its transcription. It can be removed selectively by crossing the mice transgenic for the construct with an appropriate cre mouse strain (see below) (Lakso et al., 1992). The IRES (Internal Ribosomal Entry Sequence) element enables the translation of the second cistron in a bicistronic mRNA in eukaryotes (Martinez-Salas, 1999). The STOP- IRES-d2EYFP construct is inserted into the *C-fos* gene between the BglII and Sal1 sites. How this is done is outlined below.

Methods based on recombination in E.coli has been developed for engineering large constructs like BACs and PACs (Copeland et al., 2001; Court et al., 2002). One such method that has been used for making transgenic mice was developed in 1997. The method employs in-vivo modification of bacterial artificial chromosomes in *E.coli* to make a construct in which the desired gene contained in a BAC is altered (Yang et al., 1997). The method, because it uses a BAC, has the advantage that all the sequences required for proper translation of a gene will be included in the construct and the expression will be more uniform and largely free from position effects, compared to plasmid based transgenes (Heintz, 2001). The method for insertion of foreign elements works as follows. Two sequences of homology flanking the region to be modified are cloned by polymerase chain reaction and linked together. The sequences to be inserted are cloned in between the arms and linked to a plasmid which has some special features; viz. it has a temperature sensitive origin of replication, a tetracycline selection marker and the *E.coli RecA* gene. The construct is then electroporated into *E.coli* cells containing the BAC. *RecA* mediated recombination of the construct at either of the blocks of homology into the BAC allows such cells to grow at 42˚C in medium that contains tetracycline. A second intramolecular recombination event then removes either the whole insert or just the plasmid sequences and can be selected for by placing the cells in fusaric acid containing medium, which selects for loss of the tet marker. This method is used to modify a *C-fos* containing BAC. An easier method was developed recently which involves amplifying the region to be inserted (which usually includes an antibiotic selection marker) by PCR with primers that contain short (~45bp) sequence of homology to the region to be modified (Lee et al., 2001). The BAC is placed in cells in which *gam*

and the red recombination genes *exo* and *beta* are under the control of a temperature sensitive promoter. The expression of these proteins is induced by heat shock and the PCR product is electroporated into the cells, in which they recombine. Successful recombination can be selected for by antibiotic selection. Both methods have been used in this work.

For calcium sensing, the chimeric protein ratiometric pericam is expressed under the control of a strong promoter. Pericam was constructed by Miyawaki's group in Japan by circular permutation of EYFP by cutting it near the region coding for the chromophore, attaching the N and C terminals together and cloning Calmodulin to one end and it's target peptide M13 to the other (Nagai et al., 2001). Upon calcium binding, Calmodulin binds to M13 thus reconstituting the chromophore. Ratiometric pericam has been used in cell cultures to monitor calcium levels. In the transgenic mouse described here, it is expressed under the control of the CAG promoter with the floxed stop cassette in between (Niwa et al., 1991). CAG promoter is a strong promoter that is regularly used to drive high level expression of the desired gene in transgenic mice (Okabe et al., 1997).

Cell type specificity of expression is to be conferred by crossing these mice strains to a strain expressing the cre recombinase in the desired cell type. For mitral cell specific expression, a colleague, Hans Fried is generating a strain in which the cre recombinase is under the control of the T-bet promoter. T-bet is largely mitral cell-specific, but is also expressed in TH2 lymphocytes (Faedo et al., 2002; Szabo et al., 2000). In this strain, the cre recombinase is expressed as part of a bicistronic construct using the IRES element to drive expression of cre. The double transgenic progeny form a cross between the reporter mice and the cre mice will be used to monitor activity patterns in mitral cells. Other mice strains that can be used include the CamKIIα-iCre strain.

2. Cloning of the *C-fos* gene

2.1Cloning of *C-fos* gene from Mus musculus P1 library

The mouse *C-fos* gene was cloned from a C57/Bl6 P1 total genomic library. The library was created by Dr Fiona Francis and was made available by RZPD, Heidelberg. It was available as colony macroarrays spotted as standard 4 by 4. The filters were screened with a 1.3kb mouse v-*fos* gene fragment kindly provided by Dr Ralf Heumann. Six individual clones that hybridized strongly to the probe and matched the duplicated spotting pattern were selected and ordered from RZPD. Upon further analysis by southern blotting, clone no 2 alone was found to contain the *C-fos* gene (Figure III.1). This clone was further digested with BamHI and Hind111,and run on an agarose gel. The region corresponding to 4.8 kb, the reported length of *C-fos* fragment with these enzymes that contains the coding sequence and all known regulatory elements of *C-fos* was cut out and cloned in Hind111- BamH1 digested pBluescript to generate pBS*fos.* The identity of the cloned fragment was verified by sequencing. Later a Hind111 – Not1 fragment from pBSc*fos* was transferred to pEYFP1 (Hind111- Not1 digested to remove EYFP) to generate pEY*C-fos* for further genetic manipulation.

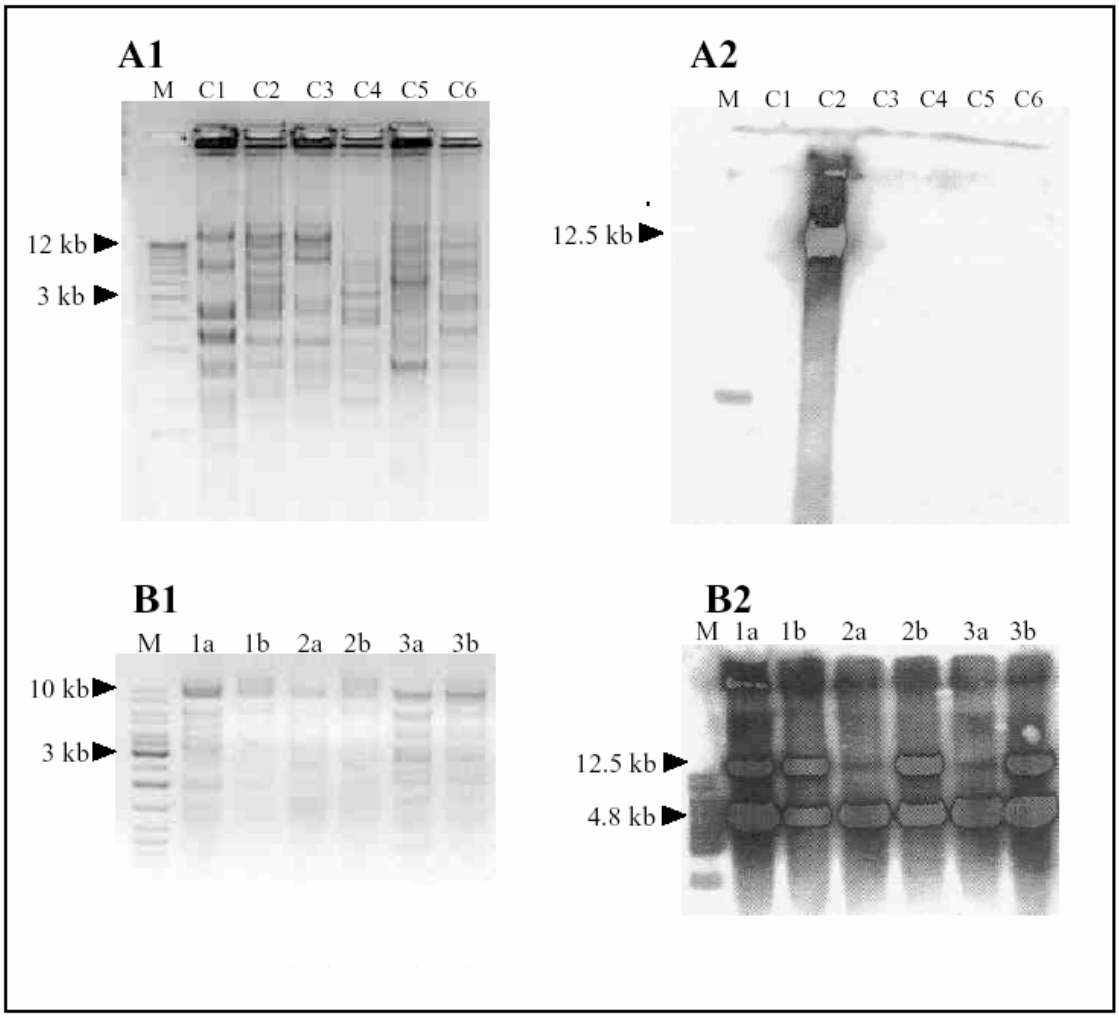


Figure III.1: Cloning of mouse *C-fos* gene from P1 and BAC libraries

A1: Ethidium bromide stained gel of six P1 clones identified by macroarray hybridization and digested with HindIII. M is marker X. C1-C6 are the P1 clones.

A2: Southern blot of A1 probed with v-fos clone. Only clone 2 is positive.

B1 Ethidum bromide stained gel of three BAC clones isolated by PCR screening of mouse BAC library and digested with BamHI-HindIII in duplicate.

B2: Southern blot of B1 with *C-fos* probe (Full length). The upper 12.5 Kb band is a result of partial digestion and the lower 4.8 kb is thee expected size with these enzymes. M is marker X and 1a-3b are the BAC clones. All three are positive.

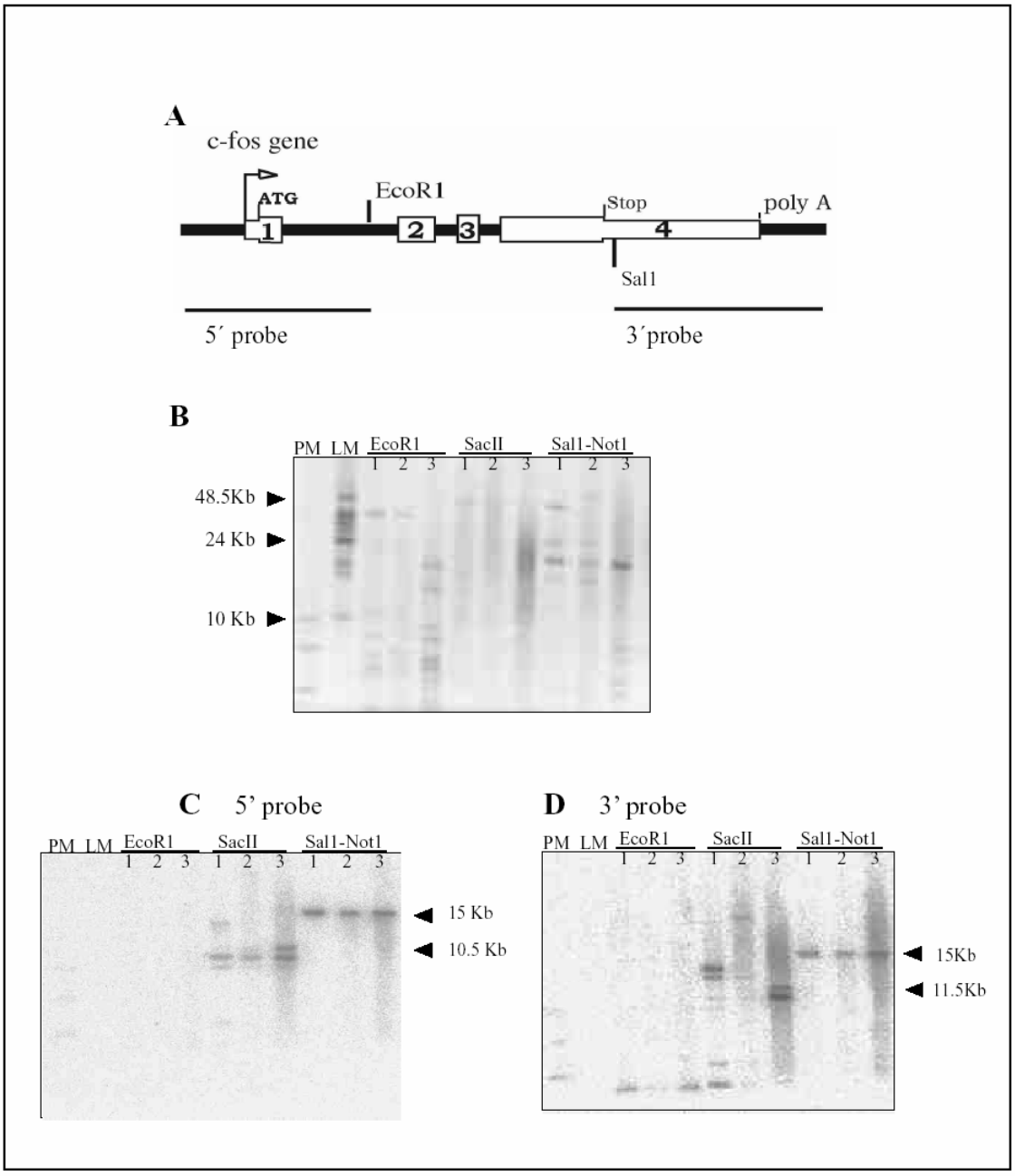
2.2 Identification of *C-fos* containing Bacterial Artificial Chromosome clones

To identify BACs containing *C-fos* gene for in-vivo BAC modification, DNA pools from a mouse BAC genomic library was screened. For this purpose, a primary pool of DNA from clones of the library was screened by PCR using primers specific for the 3' end of *C-fos* gene. In these pools, each well contained DNA from eight 384 well plates. Three pools identified by this protocol were selected and secondary pools from these were re-screened with the same method to obtain the *C-fos* containing BACs. The secondary pools contained 8 plate pools, 16 column pools and 24 row pools Three positives were obtained from each secondary pool, one for the plate, one for the row, and one for the column to uniquely identify clones containing the *C-fos* gene. Three BACS were identified from the three secondary pools .The BACs were subjected to southern blotting and probed with a *C-fos* fragment to confirm the presence of *C-fos* gene (Figure III.1).

2.2.1 Restriction mapping and southern hybridization is used to identify the C-fos BAC best suited for in-vivo modification

For BAC transgenesis, the modified gene must be ideally located in the center of the BAC so that there is sufficient length of sequences 3' and 5' to the gene that may contain regulatory elements. So, the three BAC clones obtained by screening DNA pools were further investigated by restriction mapping. The enzymes used were EcoR1, Sac11, Sal1-Not1, Not1, Pac1-Not1, BstE11-Not1, and Xho 1. The fragments were separated by Pulsed Field Gel Electrophoresis and probed with the *fos* gene fragments, 3' and 5' of the Sal1 site in *fos* gene. The most informative pattern was obtained with Pac1-Not1 where the third clone showed a bigger hybridizing fragment with both probes. This clone also showed a bigger XhoI fragment with the 3' probe (Figure III.2 G). Based on these results, BAC#3 was selected for in-vivo modification. Later on, the full sequence of this BAC was assembled by end sequencing and comparing with the mouse genome sequence

(Figure III.3). It was found to contain an insert of size 188.05 kb. There is 25 kb 5' and 158 kb 3' of the *fos* gene within this BAC clone. There is an internal Not1 site 125 kb from the *fos* gene. In addition to the *C-fos* gene, the BAC contains the *Jun dimerization protein 2* gene.


A
c-fos gene
ATG
EcoR1
Stop
poly A
1
2
3
4
Sal1
5´ probe
3´probe
B
PM LM EcoR1 SacII Sal1-Not1
1 2 3 1 2 3 1 2 3
48.5Kb
24 Kb
10 Kb
C 5' probe
PM LM EcoR1 SacII Sal1-Not1
1 2 3 1 2 3 1 2 3
15 Kb
10.5 Kb
D 3' probe
PM LM EcoR1 SacII Sal1-Not1
1 2 3 1 2 3 1 2 3
15Kb
11.5Kb

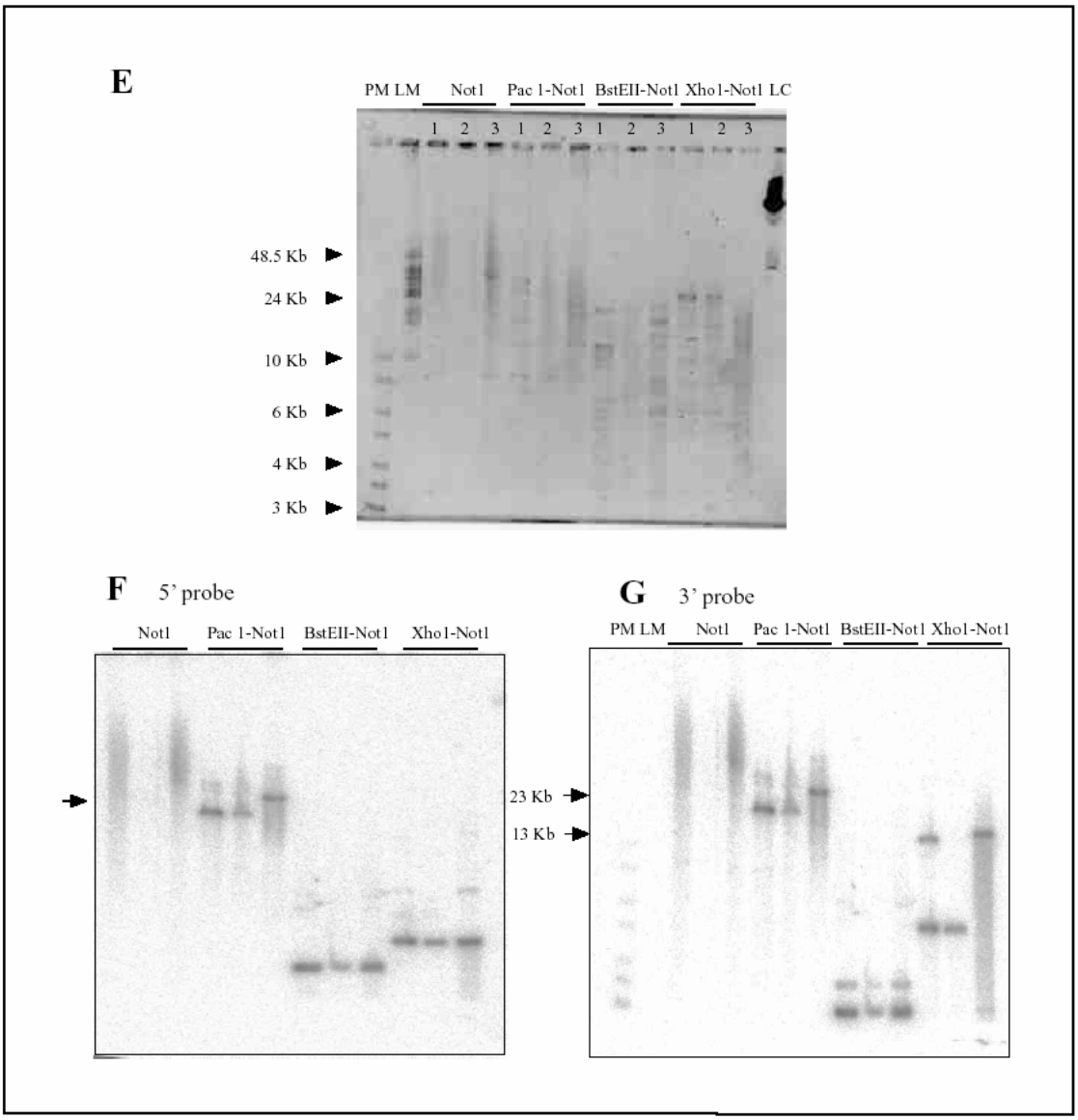

E
PM LM
Not1
Pac 1-Not1
BstEII-Not1
Xho1-Not1
LC
1 2 3 1 2 3 1 2 3 1 2 3
48.5 Kb
24 Kb
10 Kb
6 Kb
4 Kb
3 Kb
F 5' probe
Not1
Pac 1-Not1
BstEII-Not1
Xho1-Not1
23 Kb
13 Kb
G 3' probe
PM LM
Not1
Pac 1-Not1
BstEII-Not1
Xho1-Not1

Figure III.2: Identification of BAC clone best suited for in-vivo recombination.
A: The *C-fos* gene and the location of probes used for testing the BAC clones. 5'probe was taken from the region 1.5kb upstream of the EcoRI site and the 3' probe was taken from the region 1.2 kb down stream of the SalI site
B: Pulsed field gel electrophoresis of the three BAC clones identified in Fig 1 B1 with the indicated enzymes. It can be seen that the BAC clones 1 and 2 give a similar pattern and the clone 3 gives a different pattern.
C: Hybridization pattern of digests in B with 5' probe. The band hybridizing to the probe with EcoRI ran out of the gel.
D: Hybridization pattern with 3' probe. The SalI digest is incomplete.
E: Digestion pattern with more enzymes.
F: Hybridization pattern of digests in E with 5' probe. The pattern of PacI-NotI and XhoI-NotI shows difference between BAC clones 1&2 and 3
G: Hybridization pattern of gel in E with 3' probe. BAC#3 is very different and has more sequences 3' of the *C-fos* gene

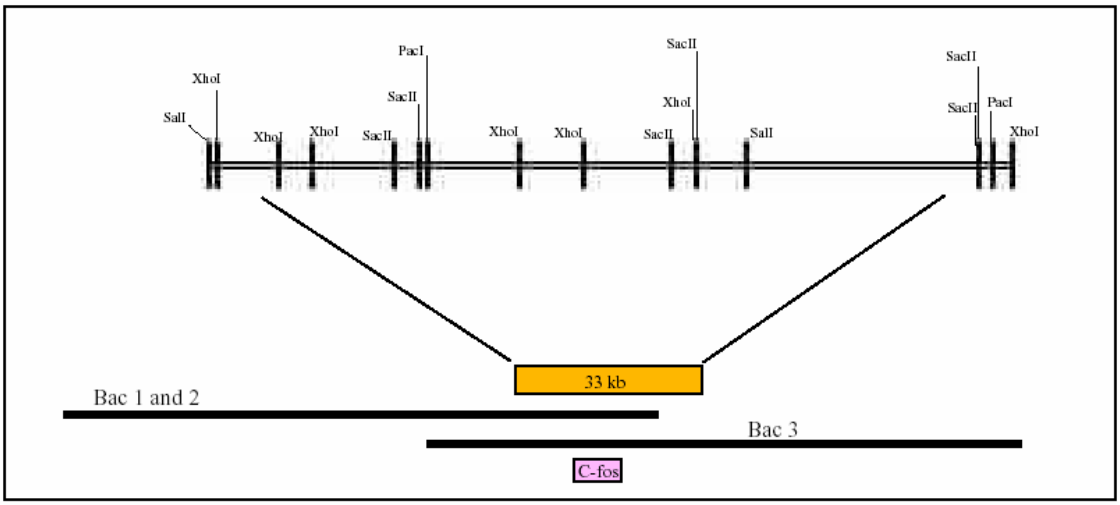


Figure III.3: The organisation of the BAC clones as deduced by restriction mapping and end sequencing. BAC I and 2 contain the *C-fos* gene at their termini. In BAC#3, the *C-fos* gene is carried more towards the middle and there is 25kb upstream and 159kb

downstream of the *C-fos* gene. A restriction map of the region relevant for the mapping experiments in Figure 2 is shown.

3. Construction of expression vectors

Various plasmid constructs were made as a part of this study with the reporters under the control of *C-fos* or CAG promoters. This section describes how they were made.

3.1 Construction of *C-fos*-STOP-EMCV-IRES-EYFP

The stop cassette was cloned by PCR from the pBS302 vector (Sauer, 1993) with both primers incorporating Xba1 sites. The PCR product was digested with Xba1, end filled and cloned into EcoRV digested pBSKS(+). The orientation of the insert was deduced by digestion with Hind111.The EMCV IRES (Encephalo Myo Carditis Virus Internal Ribosomal Entry Sequence) was amplified by PCR from IRES2EGFP vector (clontech) with the forward primer incorporating Sma1 site and the reverse primer incorporating BamH1 site. The PCR product was cloned into pGEM-T Vector and excised with Sma1 and BamH1. The fragment was cloned upstream of EYFP in pEYFP1 treated with the same enzymes sequentially. The IRES-EYFP fragment was PCR amplified with a forward primer incorporating a BstEII site and a reverse primer with Sal1 site and cloned in pGEM-T vector. pEY*C-fos* was digested with Sal1 and end-filled with klenow polymerase. It was then cut with BstEII and the large fragment containing most of *C-fos* and the plasmid backbone was purified by gel extraction. IRES2EYFP was treated with EagI, end filled and then cut with BstEII to release it from the T vector and ligated to pEY*C-fos* to generate FIY. The STOP cassette was excised from pBluescript with EcoRI

and SalI and end-filled with klenow polymerase. FIY was treated with EcoRI, dephosphorylated and end-filled with klenow polymerase. It was then blunt end ligated to the stop cassette. (Figure III.4 B) All the constructs were tested by restriction digestion and/or sequencing at each step.

3.2 Construction of *C-fos*-STOP-GtxIRES-EYFP

A new synthetic IRES element which is made of multiple copies of a 9 bp sequence from a cellular mRNA (Gtx) was reported to enhance the translation of the cistron downstream of it several fold over control level. This element was tested in this study. To make a construct with Gtx IRES under *C-fos* control, it was necessary to convert the 5X Gtx IRES (kindly provided by Vincent Mauro) to 10X, which showed maximum enhancement of translation in the original study. The photinus luciferase in GtxBRPh plasmid was replaced with EYFP as follows: pEYFPI was cut with NotI and endfilled. EYFP was released from it by treating with NcoI. GtxBRPh was cut with BamHI, end filled and then Photinus luciferase was removed by cutting with NcoI. EYFP was then ligated to GtxBRPh.. GtxEYFP was subcloned by releasing it from the parent plasmid with EcoRI and SalI and ligating to pBluescript treated with the same enzymes. It was linearised with EcoRI and the oligonucleotides GIR-S and GIR-A were annealed together and ligated to it to generate Gtx(10X)EYFP. GtxEYFP was amplified by PCR with a forward primer incorporating BstEII site and ligated to pEY*C-fos* treated with BstEII and SalI. The stop cassette was cloned to this construct as described in the section 3.1(Figure III.4.B).

3.3 Construction of CAG-STOP-RmPc

Pericams are a new generation of calcium sensing proteins that change fluorescence upon calcium binding. For cell type specific expression in mice of ratiometric pericam (RmPc)

was cloned under the CAG promoter with the floxed stop cassette in between. Ratiometric pericam was excised from pcDNA3 with BamHI and NotI and cloned into pBluescript treated with the same enzymes. CAG promoter was subcloned into pGEM5Z (+) with SalI and PstI. RmPc was excised out from bluescript with EcoRI and cloned into the EcoRI site in CAG. The stop cassette was excised with EcoRI and SalI, end filled and cloned into the SmaI site in CAGRmPc to generate CAG-STOP-RmPc (Figure III.4 C).

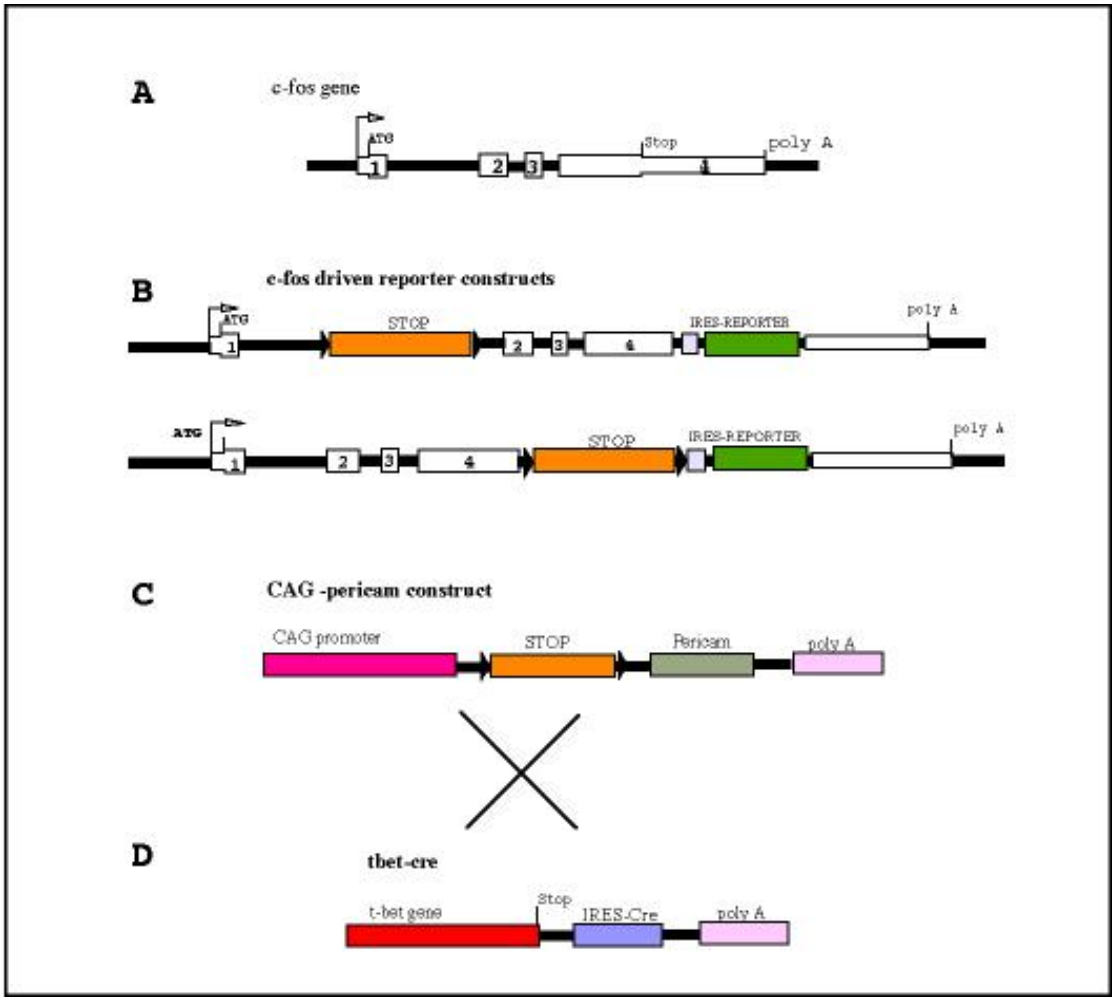


Figure III.4: Schematic of the *C-fos* and pericam and the *T-Bet* constructs. The *C-fos* gene (A) was modified as shown (B) with the stop cassette in intron one or exon four.

The IRES-d2EYFP cassette was always placed in the exon four. C: The CAG-STOP pericam construct.
D: Schematic of the *T-bet*-cre construct. White boxes are exons of *C-fos* and black lines are introns

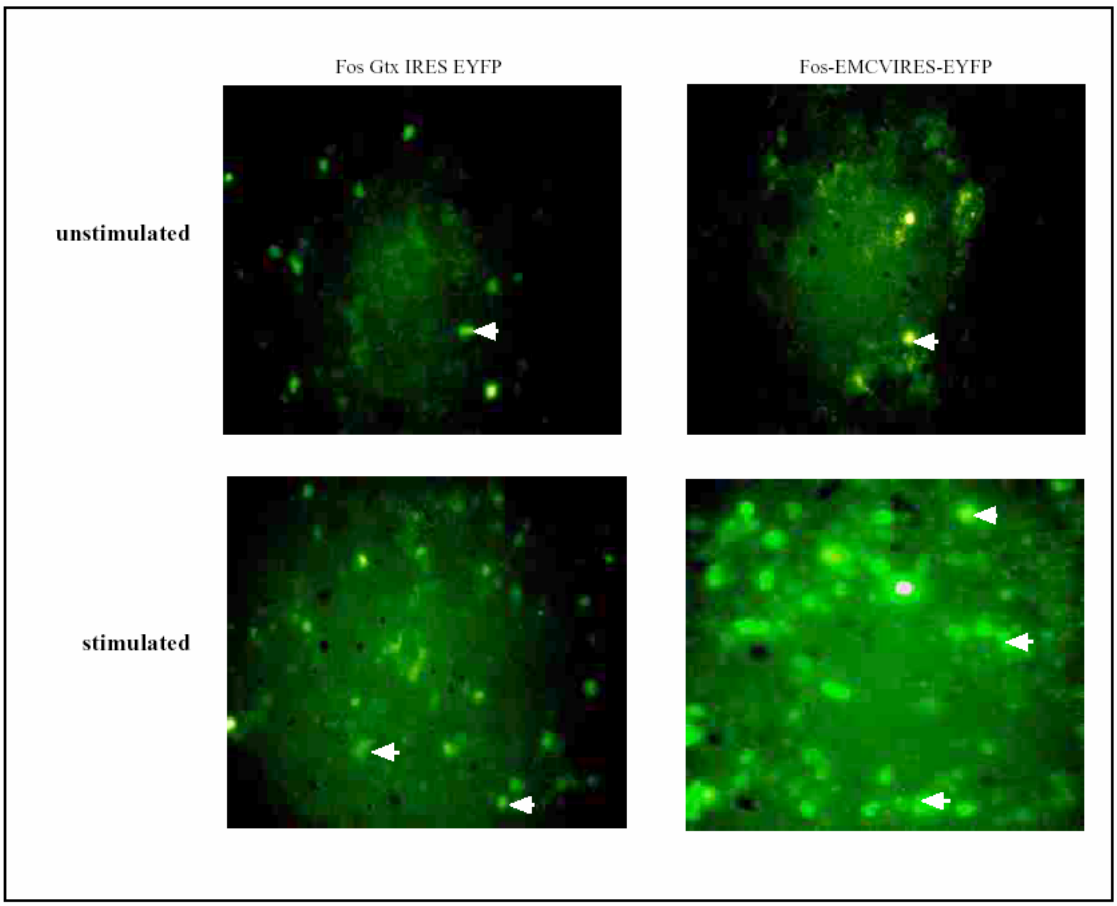


Figure III.5: Testing of Fos STOP-IRES-D2EYFP constructs in HEK cells in culture. Two different IRES elements viz. GtX IRES and EMCV IRES were used in these constructs .The STOP cassette is in intron one. Control cells (unstimulated after transfection) showed little EYFP expression while cells in which the *C-fos* gene was stimulated with TPA (a Phorbol ester) showed robust expression when EMCV IRES was used but not with Gtx IRES. Arrows indicate cells.

4. Plasmid constructs were tested by transfection of cells in culture

To test if the various elements assembled into the constructs described in section 3 function as desired, they were transfected into HEK293 cells in culture. About 500 ng of plasmid were used per well of a 24 well plate and transfection was done with lipofectamine dye. All the constructs were transfected with and without a cre expression vector in parellel. Expression is expected in those cells that received the constructs along with the cre plasmid. To stimulate the *Fos* promoter (which is not constitutive), 25nM TPA, a phorbol ester was added to the cells after transfection. The expression of the reporter genes was followed by fluorescence microscopy. EYFP expression was detected in *Fos* constructs as shown in Fig III.5. But it was noticed that EYFP expression was not dependent on the cotransfection of the cre expression vector for the construct shown in Fig II.4 B. Expression was also seen in those wells that did not receive the cre plasmid. The low-level exprerssion seen in unstimulated cell is probably because of the stress of transfection that may stimulate *C-fos*. The positioning of the stop cassette within an intron probably affected its proper functioning. The CAG-STOP-RMPC construct was found to function as expected (data not shown). It was concluded that these *Fos* constructs are not suitable for modification of BACs as they are.

5. Construction of *Fos* recombination cassette

5.1 Homology regions A and B were amplified by PCR and linked together.

A new *Fos* transgenic mouse line with LacZ as reporter was reported recently. In this strain, LacZ was expressed as a fusion protein with *C-fos* by fusing it at the BglII site in *C-fos*. Taking cue from this construct, a new *Fos* recombination cassette was made as

shown in Fig 6.Two homology arms of about 500 bp each for *RecA* mediated recombination were cloned from the region upstream of BglII (homology A) and downstream of SalI (homology B). Thus, the region between BglII and SalI is deleted in this construct, generating a truncated *C-fos* protein. The forward primer for homology A and reverse primer for homology B had SalI recognition sites built into them so that later, the cassette can be cloned into pSV1*RecA*. Further, the 5' ends of the homology A reverse primer and the homology B forward primer had each a 15bp region complimentary to each other. In addition this region contained a stop codon in frame to the *C-fos* reading frame and an XhoI site. The homology A and B regions were amplified seperately by PCR. Equal amounts of the PCR products were mixed together and amplified with the outer primers (homology A forward and homology B reverse) to generate the fused homology regions A and B which was cloned into the pGem-T vector (Figure III.6).

5.2 STOP-IRES-d2EYFP was built piecewise and cloned in between homology A and B

At this point of time, it was decided to d2EYFP as the reporter for *C-fos* because it turns over faster and at a rate similar to that of the native *C-fos* protein. Thus the reporter is expected to mimic the expression pattern of *C-fos* at all levels (mRNA and protein) and the mice may be used for multiple imaging sessions. d2EYFP and EMCV-IRES were cloned into the pGem T vector by PCR from IRES2EGFP and pd2EYFPN1. d2EYFP was excised form T-vector with BamHI and NotI and cloned into pGemT-IRES cut with the same enzymes. IRES2-d2EYFP was excised with SmaI and NotI and cloned into pBS STOP cut with the same enzymes to generate STOP-IRES2-d2EYFP.It was then excised with SalI and cloned into the homology AB digested with XhoI. STOP-IRES2-d2EYFP was also cloned into the SalI site of *C-fos* gene to generate the construct shown in II.5C.It was realised that d2EYFP was one nucleotide out of frame with respect to IRES2. To

rectify this, it was treated with AgeI and the protruding four nucleotides were removed with mungbean nuclease and self ligated and verified by sequencing The whole cassette was then excised with SalI and cloned into pSV*RecA* cut with SalI (Figure III.6).

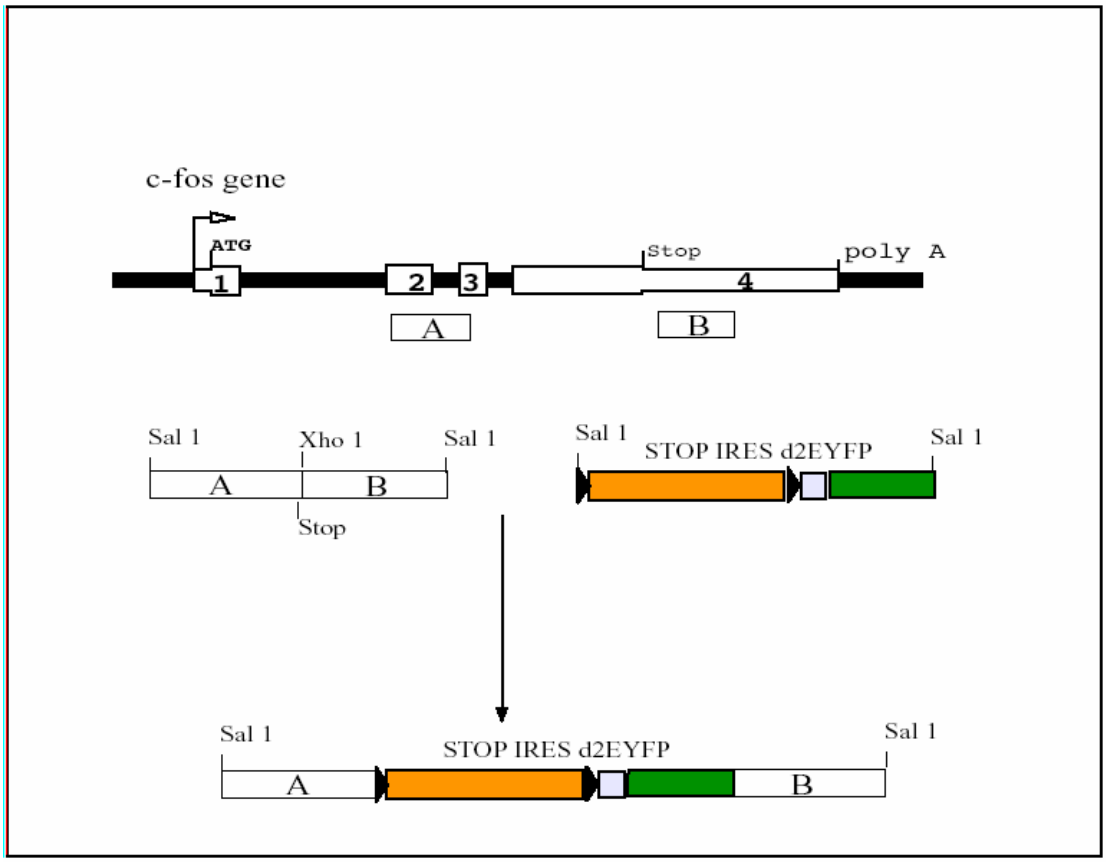


Figure III.6: Construction of the new recombination cassette for RecA mediated BAC modification. Regions of homology were cloned from the indicated parts of the *C-fos* gene. The homology A forward primer contained a SalI site at the 5' end and the reverse primer a stop codon and an XhoI site included in a 15bp region of homology to the 5' end of the homology B primer. The homology B reverse primer contained a SalI site at its 5' end. The two fragments were amplified by PCR. The A+B fragment was amplified by mixing the A and B fragments and amplifying with the outer primers. The

STOP-IRES-d2EYFP cassette was cloned into the XhoI site and the whole construct was cloned into the SalI site of pSV.1 RecA vector.

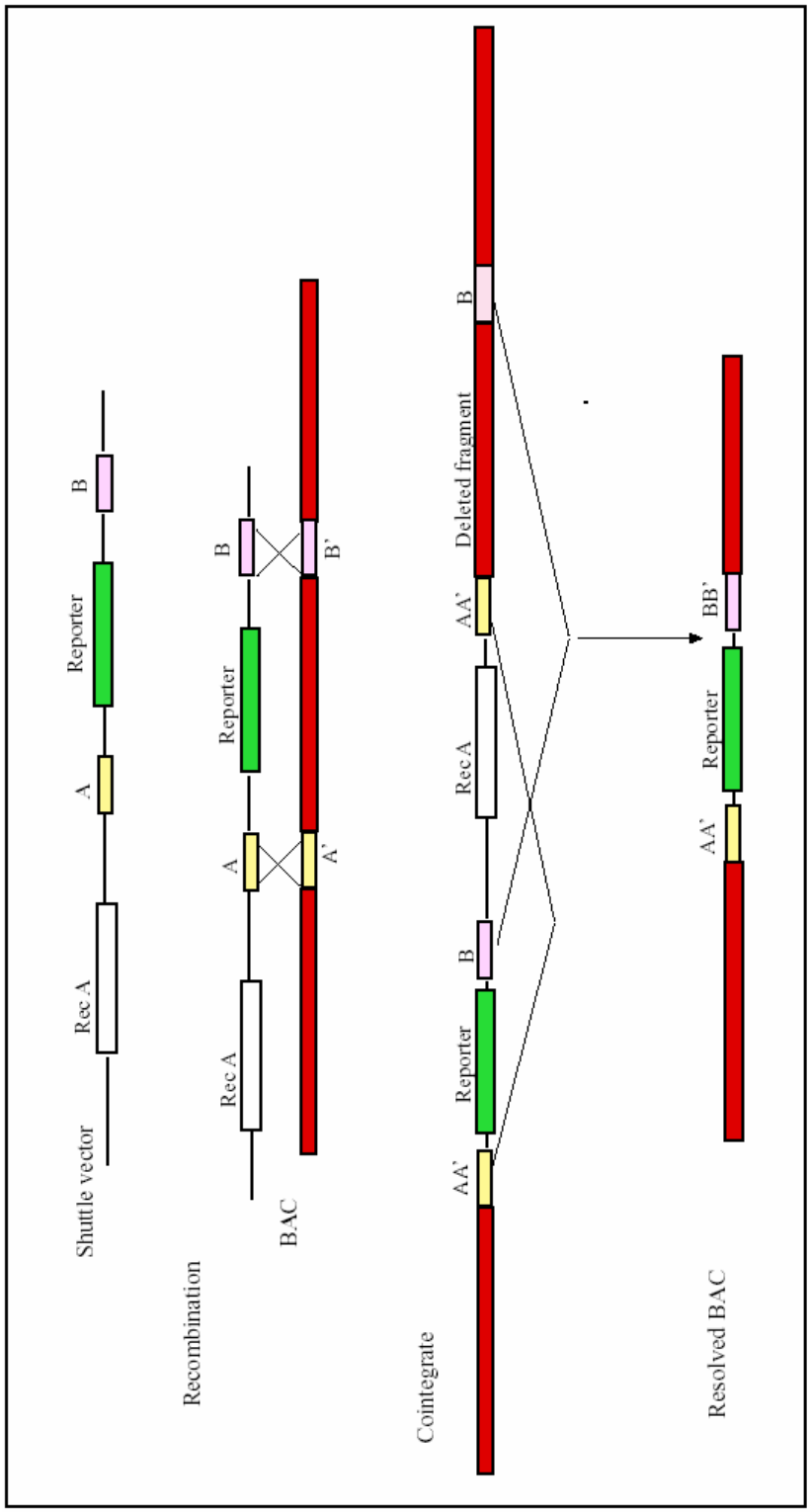


Figure 7:The different steps in in- vivo BAC modification (insertion). The sequences to be inserted are flanked by two regions of homology to the site of modification and cloned into the Psv Rec A vector. The vector is electroporated into E.coli harbouring the BAC to be modified Recombination at homology region A or B inserts the plasmid into the BAC.The BAC resolves by intramolecular recombination throwing out either the whole plasmid or sequences except the desired modification

6. Modification of Bacterial artificial chromosomes in-vivo

Restriction enzyme mediated modification of genes breaks down when large constructs like BACs have to be dealt with, because, they have multiple recognition sites for most of the restriction enzymes. To circumvent this limitation, recombination based methods were developed which do not require the use of restriction enzymes for modification of large constructs. Methods based on two important recombination systems in *E.coli* are described in this thesis; one based on the *RecA* system and the other based on the Red-Gam system.

6.1 Modification of *C-fos* gene carried in a BAC by *RecA* mediated recombination in *E.coli*

One of the earliest such methods utilizes the *RecA* gene of *E.coli* for modifying BACs. In this study, I used this method for modifying the *C-fos* gene carried in a BAC.

6.1.1 pSV.1RecA carrying the recombination cassette integrates into the BAC at the C-fos locus

The recombination cassette (section 5) was electroporated into *E.coli* cells harbouring the BAC chosen for recombination. An aliquot of the cells were plated on LB plates containing tetracycline and chloramphenicol as selection markers and incubated overnight at 30˚C. Several colonies which received the plasmid were found to have formed. Six of these were picked and diluted in 1ml LB. medium and an aliquot was spread on plates containing tetracycline and chloramphenicol. These plates were incubated at 43˚C overnight upon which a few big colonies were found to have formed on a background of minute satellite colonies. These are the cointegrates, cells that have

integrated the plasmid at the *C-fos* locus by recombination at either homology A or B. Only cointegrates can grow at 43˚C in medium containing tetracycline, because, pSV.1 *RecA* is replication deficient at this temperature and the only tet resistant cells are those that have incorporated the plasmid into the BAC. Twenty colonies were selected from among the cointegrates for further investigation by southern blotting. BAC DNA prepared from them was subjected to digestion with BamHI and southern hybridization with homology arm B. As shown in Fig III.8 A, in addition to the 11kb band of the *C-fos* locus in BAC, some clones showed a 2.3 kb band that arises from the recombination cassette. These are the clones that have correctly recombined.

6.1.2 Incubation in fusaric acid containing medium selects for resolved cointegrates

After the correctly recombined cointegrates were identified, the respective colonies were streaked out on plates containing chloramphenicol as selection marker and grown overnight at 43˚C. In some of these cells, the pSv.1*RecA* plasmid is removed from the BAC by a intra-molecular recombination event. The plasmid is then lost from the cell because it cannot replicate at 43˚C and since there is no tetracycline in the medium, such cells also can grow. Two types of BACs can result from this second recombination event; those that have a modified *C-fos* locus and those that have wild type *C-fos* locus. A modified locus can result only if the second recombination event involves a different homology arm from the first one. The next step is to select for resolved cells. This was done by streaking out these cells in medium containing chloramphenicol and fusaric acid. Only cells that have lost the tet resistance gene can grow in the presence of fusaric acid and so this step selects for resolution of the BAC. A few dozen colonies were observed to have grown in the presence of fusaric acid after extended incubation at 37˚C for 3 days. Twenty of these colonies were selected and BAC DNA was prepared from them. It was digested with BamHI and as shown in Fig III.8 B some of the clones show a 2.3kb band with the homology arm B probe, which is the signature of correct modification.

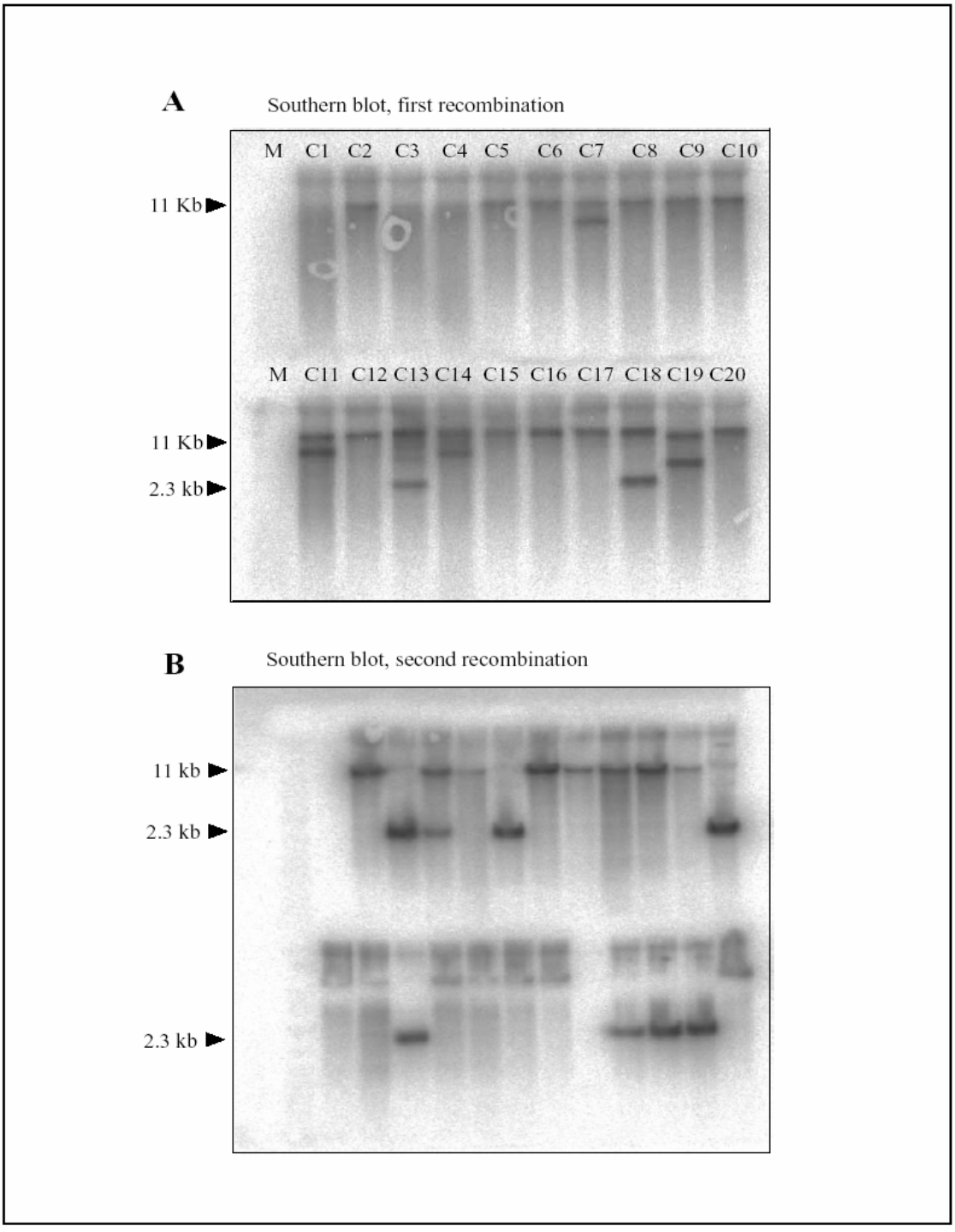

A
Southern blot, first recombination
M C1 C2 C3 C4 C5 C6 C7 C8 C9 C10
11 Kb
M C11 C12 C13 C14 C15 C16 C17 C18 C19 C20
11 Kb
2.3 kb
B
Southern blot, second recombination
11 kb
2.3 kb
2.3 kb

Figure III.8: RecA mediated BAC modification. The pSV.I RecA vector carrying the recombination cassette integrates into the BAC by intermolecular recombination at homology A or B. Southern blot of BamHI digested BAC DNA with homology B probe reveals the two bands in the cointegrate (A). Resolution occurs by intramolecular recombination and is revealed by a single band at 2.3kb in modified BACs and 11kb in unmodified BACs (B)

6.1.3 The fidelity of recombination is assessed by PCR and restriction mapping.

Because of the presence of *RecA* gene in the cells for an extended period of time, there is a chance that the BAC undergoes undesirable rearrangements. It is important to make sure that such rearrangements have not taken place in BACs used for generating transgenic mice. This was done by extensively investigating the BACs by PCR and restriction mapping. PCR with the primers directed against the inserts showed their presence and integrity. Further PCR reactions were done with one primer within the insert and the other binding to a region flanking the insert outside the homology arms. (Figure III.9 A & B). These reactions suggested that correct recombination has taken place and that there has been no undesirable modifications at the *C-fos* locus. Further, the chosen clones were subjected to digestion with various enzymes and the resulting fragments were separated by gel electrophoresis. They were then blotted and hybridized with homology arm B probe. As shown in Fig III.9, the recombined clones show larger bands with BstEII and SacI, because the deleted region contains a site each for these enzymes. With BamHI, they show a smaller band because a BamHI site is added to the locus by the insert (Figure II.9 C). The digested BAC DNA was also run alongside unmodified BAC in a pulsed field gel and the pattern looked similar with all the enzymes tested (the difference due to insert is not easily noticeable). Over all, the PCR and southern data strongly support the conclusion that the BAC has been correctly modified and that there has been no undesirable modifications.

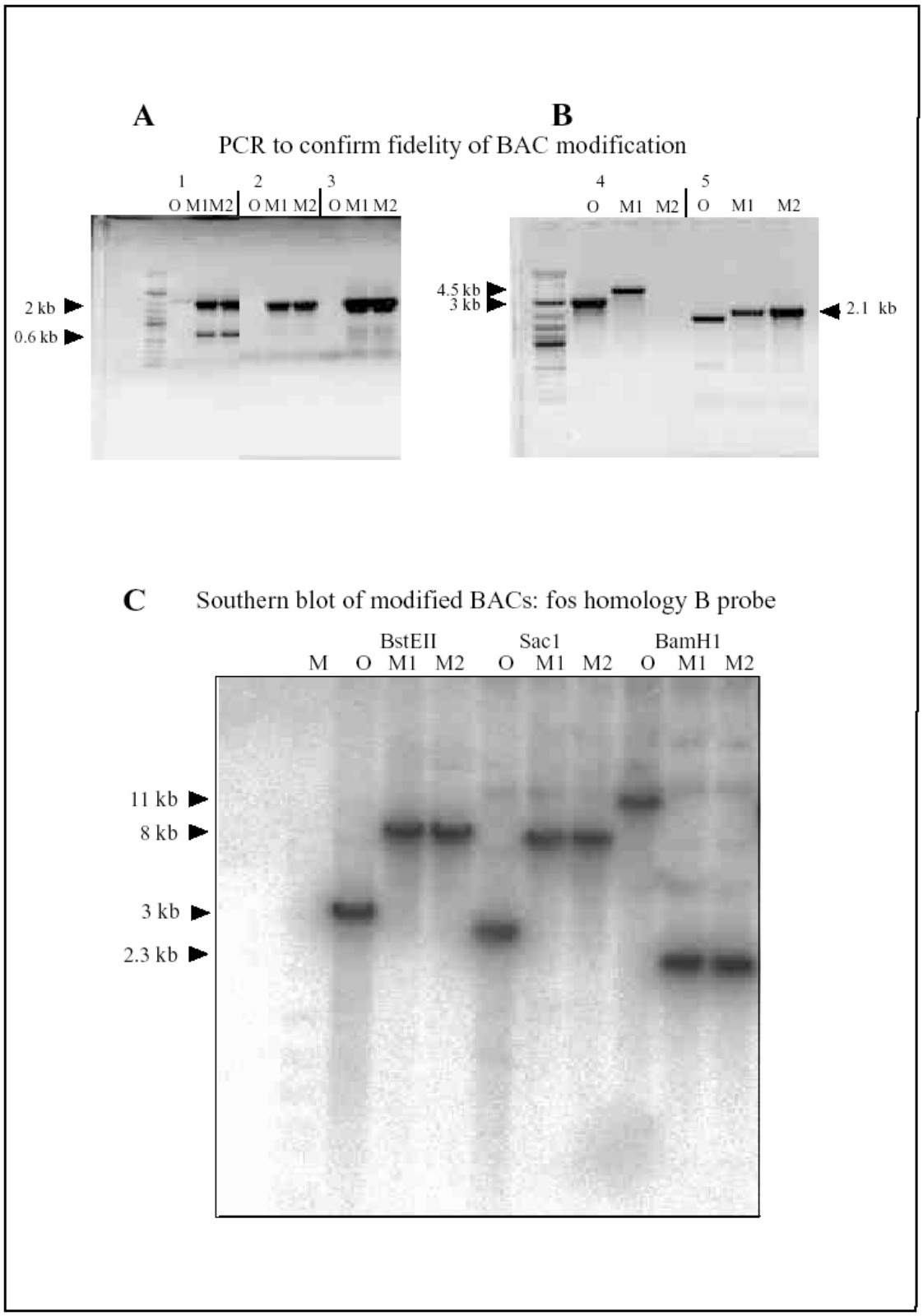

A
PCR to confirm fidelity of BAC modification
1
2
3
O M1M2
O M1 M2
O M1 M2
2 kb
0.6 kb
B
4
5
O M1 M2
O M1 M2
4.5 kb
3 kb
2.1 kb
C
Southern blot of modified BACs: fos homology B probe
BstEII
Sac1
BamH1
M O M1 M2 O M1 M2 O M1 M2
11 kb
8 kb
3 kb
2.3 kb

Figure III.9: Assessing the fidelity of BAC modification by PCR and southern blotting. A: 1-Primer against loxP sequences and a primer outside the region of recombination next to homology A. 2-Primers against full length STOP cassette. 3. Primers against full length IRES-EYFP. 4- Primers just outside homology A and homology B. 5. Primers against 5' end of IRES and just outside homology B. O is original BAC M1 is modified BAC #3, M2 is modified BAC #12 (Fig III.8). The bands shown by modified BACs indicate correct modification.

B. Restriction mapping also confirms correct modification. One BstEII and SacI site each are removed from the *C-fos* locus by modification resulting in larger bands with these enzymes. A new BamHI site is introduced, giving rise to a smaller band.

6.2 Gel permeation chromatography failed to separate backbone from insert

The BAC clone #3 resulting from resolution (Figure III.8) was selected for microinjection based on the PCR and southern data. It was necessary to remove the backbone of the BAC before microinjection because it contains loxP sites that can give rise to undesirable recombination's with the loxP sites in the stop cassette when crossed to cre expressing mice. The recommended method for removing the backbone is digestion with NotI followed by gel permeation chromatography with sepharose CL4B beads. This column material was not found to be optimal for separation, so another column material, sephacryl S-1000 which has a theoretical cut off of 20kb was selected. The result with this material is shown in fig III.10. There were two peaks of DNA upon elution with the injection buffer, a major peak that eluted first and contained the bulk of the DNA but without efficient seperation of backbone and insert and a second minor peak that apparently contained only the insert (Figure III.10 A). Aliquots from both peaks were subjected to pulsed field gel electrophoresis and it was found that the DNA from major peak was degraded while the minor peak contained insert that looked intact. (Figure III.10 B) DNA from this peak was chosen for microinjection.

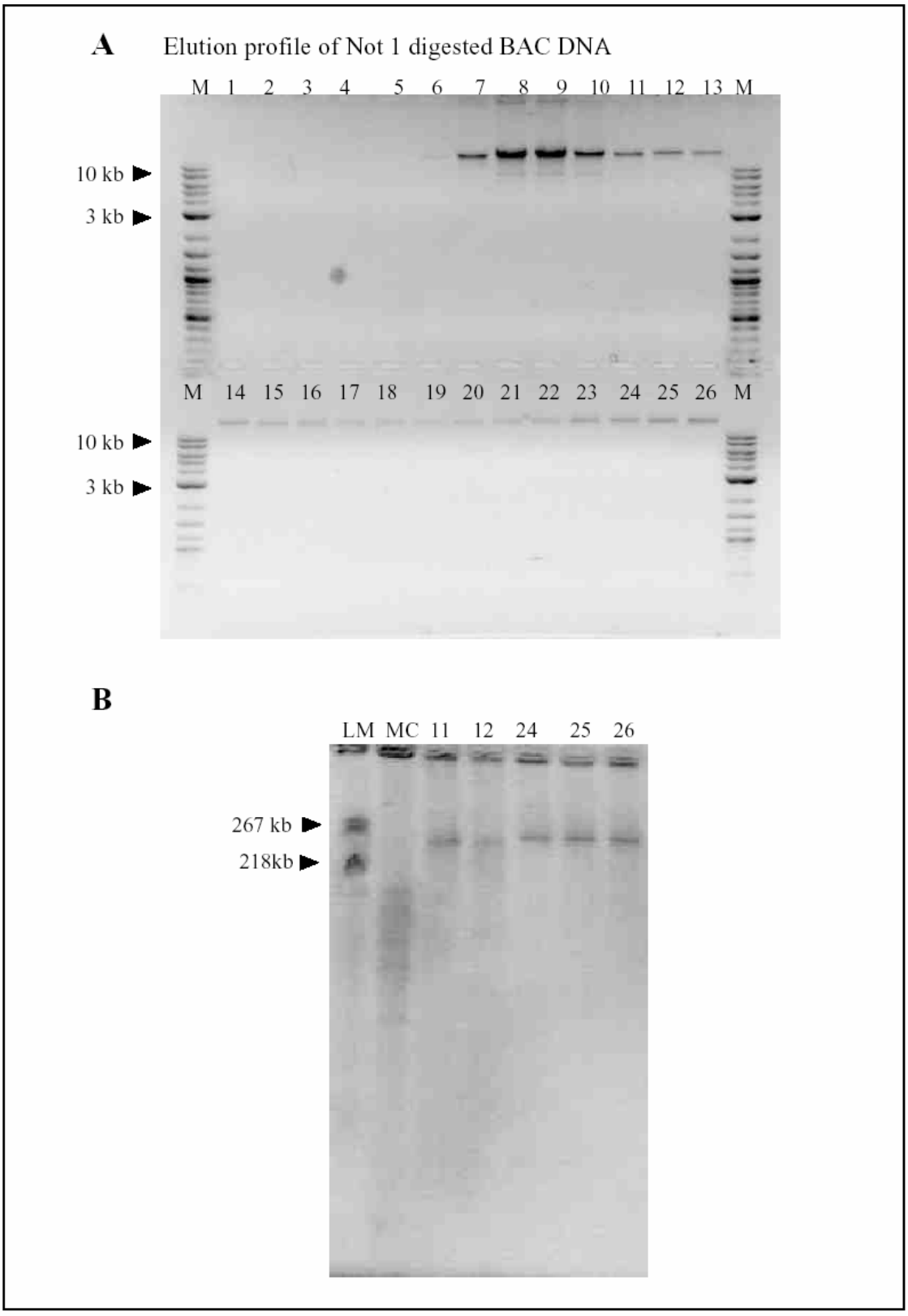

A
Elution profile of Not 1 digested BAC DNA
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
10 kb
3 kb
M 14 15 16 17 18 19 20 21 22 23 24 25 26 M
10 kb
3 kb
B
LM MC 11 12 24 25 26
267 kb
218kb

Figure III.10: Fractions from gel permeation chromatography separated by routine agarose gel electrophoresis (A) and Pulsed Field Gel Electrophoresis (B). Note the second peak in fractions 23-26 in A. Pulsed filed gel electrophoresis shows that the DNA in the first peak is degraded while that in the second peak is intact.

6.3 BAC modification by Red-Gam Mediated recombineering

The Red-Gam system is derived from the phage lambda of *E.coli.* Gam inhibits the Dnase activity of RecBCD on linear DNA and the Red proteins Exo and Beta generate recombination activity. This system can effect recombination with just 40-50 nucleotides of homology on either side of the insert and its variants can be used to subclone DNA from large constructs also. This system was used in this study to remove the wild type loxP site in the BAC so that intact BACs can be used for making transgenic mice.

6.3.1 Red-Gam system utilizes PCR primers with 40 bp homology regions at the 5' ends

The first step in Red-Gam recombination is to design PCR primers for the amplification of PCR products that are used in modification. In this study, the wild type loxP site and the *Sac*B gene in the BAC vector (pBACe3.6) was replaced by the kanamycin resistance gene. PCR primers were designed with their 3' end complimentary to the kanamycin gene in pL451 and 5' end 40 nucleotides complimentary to the regions flanking LoxP (forward primer) and the *Sac*B gene (reverse primer) in the BAC. The recombination cassette was generated by PCR amplification from pL451 using these primers.

6.3.2 The PCR product undergoes recombination with the BAC in heat shocked DY380 cells

Competent cells for electroporation of the PCR product are specially treated. They are subjected to heat-shock for induction of the Red-Gam proteins before washing in sterile water. The PCR product is introduced in to these cells by routine electroporation and it recombines with the BAC at the appropriate site to generate the modification. Recombinant cells were selected on media containing Kanamycin and chloramphenicol. Several methods were used to assess the fidelity of recombination. A PCR reaction with the forward primer used for recombination and a reverse primer binding to a region flanking the site of modification gave a product of the expected size (Figure III.11 A). Also, restriction mapping showed that the gross pattern of digests was not different between the original and modified BACs. Southern blotting with the Kanamycin cassette as probe showed that it had inserted in the BAC and showed the expected polymorphisms with restriction enzymes. (Figure III.11 B)

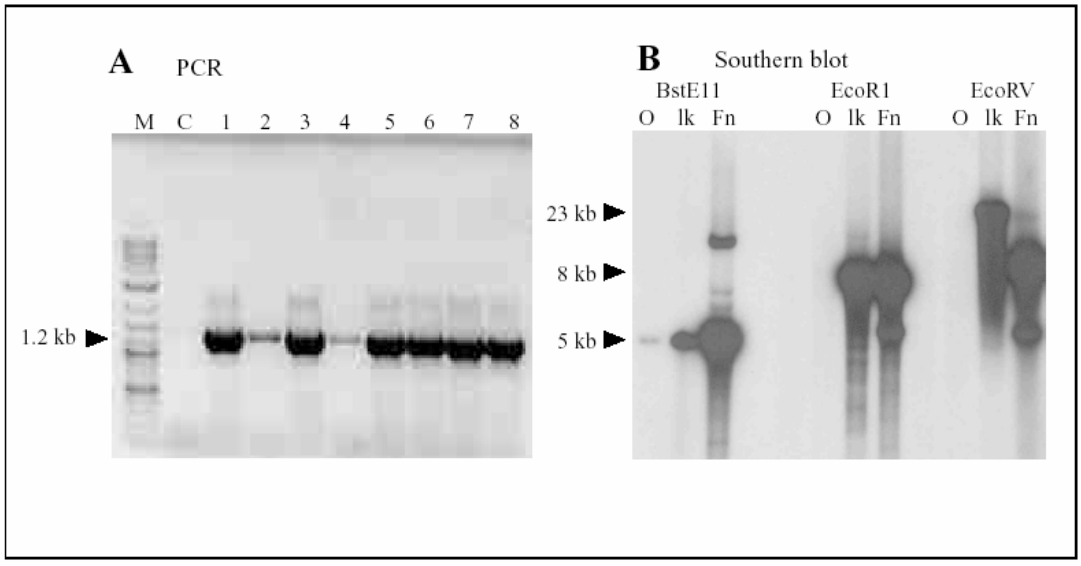


Figure III.11: Analysis of BACs from coupland recombination.
A: PCR with a primer directed against the kanamycin cassette and another to a region flanking the modified region gives a correct sized band of 1.2kb in eight BACs randomly picked after recombination (1-8) C is unmodified control BAC. Southern blotting with kanamycin cassette as probe reveals it's presence in modified BACs at the correct

molecular weight. lk: loxP exchanged against Kan cassette. Fn**:** A neomycin cassette inserted next to *C-fos*. This BAC was intended for recombination in ES cells, but was not used.

6.4 Three rounds of microinjection were carried out for creation of *Fos*-d2EYFP mice

The BAC constructs were injected into fertilized oocytes. Three rounds of microinjection had to be carried out for the generation of the *Fos*-d2EYFR strain. In the first round, DNA that eluted in the second peak from the Gel-permeation column was used. Fifty progeny were obtained from this round of microinjection and all turned out to be negative for the BAC as determined by southern blotting with stop cassette as the probe. Following this, the loxP site in the BAC was removed by Red-Gam recombination and intact circular BAC was used for microinjection. In this round, only thirty-three progeny were obtained and none were positive for the transgene. A third attempt was made and using the same BACs as in the second attempt. Forty-nine progeny were obtained and one of them turned out to be positive for the transgene (Figure III.12 A). This mouse is being crossed to wild type C57/BL6 mice and the progeny were genotyped by PCR with primers against the STOP cassette. Four out of eleven were positive from those analyzed so far (Figure III.12 B).

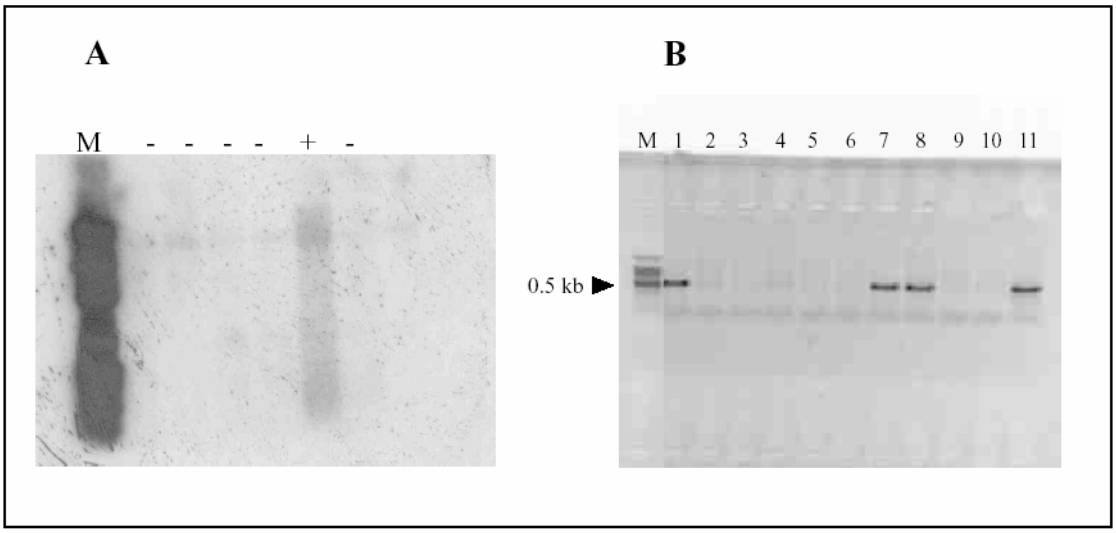


Figure III.12: Establishment of Fos-STOP-IRES-d2EYFP strain. Southern blot of genomic DNA from F0 (founder) generation revealed that one of them (+) carried the transgene (A). Four/eleven of progeny from a breeding of this mouse to Bl/6 were positive for the transgene as shown by PCR with primers directed against the STOP cassette (B).

7. Generation and breeding of Ratiometric Pericam transgenic mice

Calcium transients at neuronal terminals are important indicators of neuronal activity. Both axonal and dendritic termini have strong calcium currents passing through voltage gated and ligand gated calcium channels and intracellular stores also release calcium. Monitoring Ca^{2+} influx in the glomeruli upon stimulation with odors in mice in which the olfactory receptor neurons are labeled with Ca^{2+} sensitive dyes is an established technique in our lab. Cells that are inaccessible to dye labeling, like the mitral cells can be labeled genetically by cell type specific expression of calcium sensing proteins like

pericams. Here I chose to express ratiometric pericam specifically in the mitral cells by exploiting the cre-loxP system in mice.

7.1 Pericam transgenic mice were generated by two rounds of microinjection

The construct shown in fig III.4 C was excised with NsiI and NotI from pGem5 (Z) + and microinjected into pronuclei of mice zygotes. Two rounds of microinjection were carried out to generate this strain. In the first round, 21 progeny were obtained. DNA was isolated from tail biopsies, digested with XbaI and subjected to southern blotting. Three mice from this generation (F0) turned out to be positive for the pericam transgene (Figure III.13 A). Thirty-nine progeny were obtained in the second round of microinjection and southern blotting with EcoRV digesion revealed that five of these were transgenic (Figure III.13 B). All the founder mice were bred to C57/BL6 mice and the progeny were analyzed by southern blotting with pericam probe after digestion with EcoRV.

7.2. Six transgenic lines were derived from the founders

The founder mice were bred to wild type C57/BL6 mice and the progeny were analyzed for germline transmission of the transgene by southern blotting. Germline transmission could be established for six founders. One of these founders, CSRSun 9, segregated to form three sublines derived from three progeny that had different southern hybridization patterns. Thus, in practice there were eight different lines each of which had unique sites of transgene integration and copy number. The copy number varied from apparently one for CSR Sun 0 to tens for CSR Sun 9 as seen by southern blot. An attempt was not made to assess the copy number of each line accurately because it is known that transgene expression is not directly dependent on copy number and it is difficult to find out exactly.

All lines were maintained as heterozygotes as breeding to make them homozygous for the transgene might affect their viability.

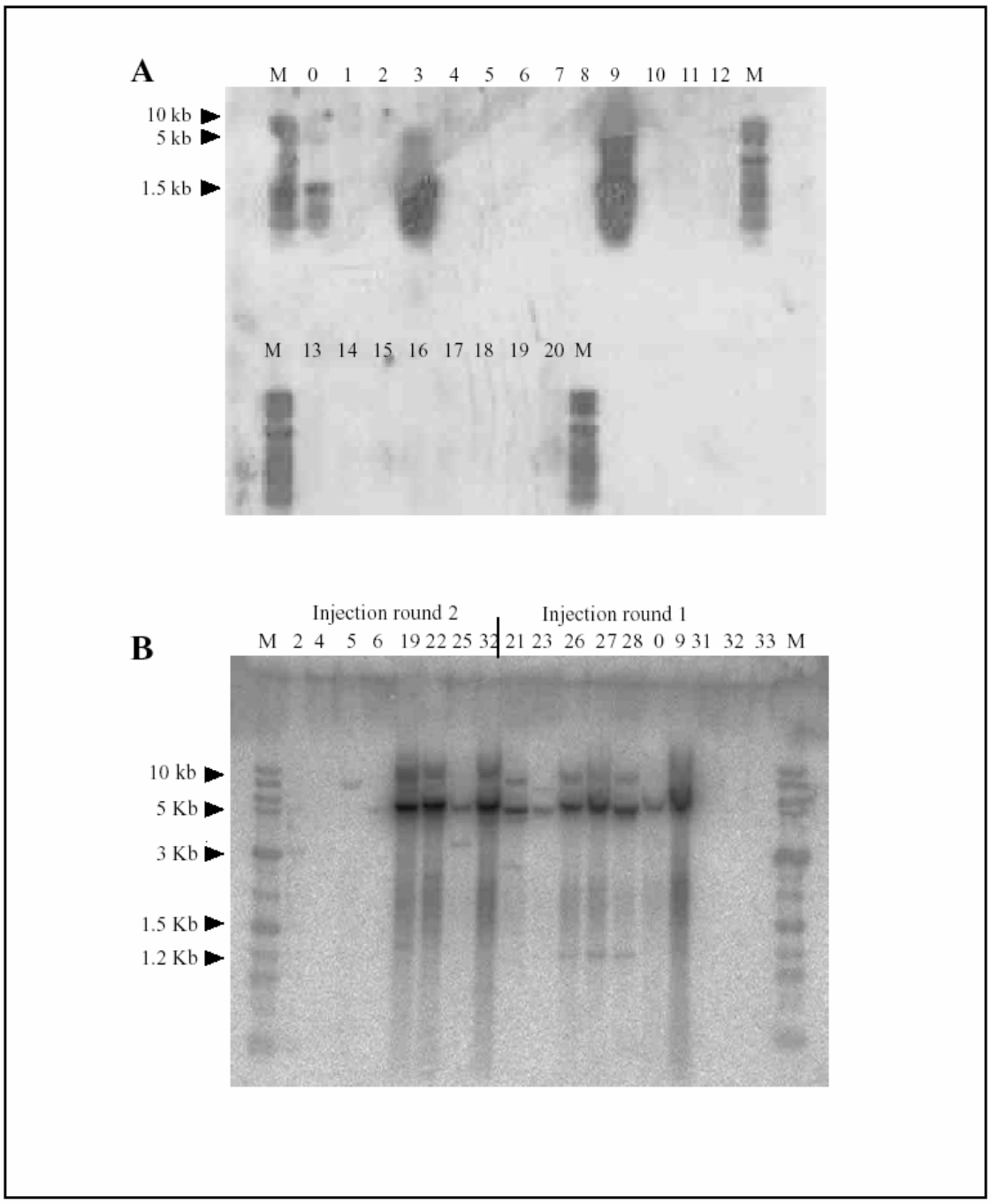


Figure III.13: Generation of pericam transgenic mice. Genomic southern blot after digestion with XbaI reveals that three of the twenty-one F0 mice are positive for the transgene (A). More mice are generated by a second round of microinjection (B) In this case digestion is with EcoRV. Lines that were established are: CSRSun 0 (0 in A), CSR Sun 9 (A). CSRSun 54 (6 in B), CSR Sun 67 (19 in B), CSRSun 70 (22), CSRSun 73 (25), and CSRSun 80 (32). Note that EcoRV that cuts once with in the injected fragment gives a main band of unit size and two off-sized bands of different sizes in different lines. This points to tandem integration of transgenes.

7.3 Breeding to CMV-Cre mice did not delete the stop cassette

After establishing different lines, it was necessary to cross them to a cre expressing line to check for pericam expression. The cre recombinase catalyzes an intramolecular recombination between the two loxP sites in the stop cassette leading to a deletion of the stop signal. Relieved of the transcriptional blockage, the ratiometric pericam gene should then be expressed. All eight mice lines were crossed to a CMV–Cre mice strain that expresses cre recombinase under the CMV early promoter. This strain has been shown to express the cre transgene from the early embryonic stage and the transgene is thought to be X-linked (based on the inheritance pattern) (Schwenk et al., 1995). Cre homozygous females were chosen for breeding to pericam positive males and the progeny had to be genotyped only for pericam because all progeny are heterozygous for cre. And, because the CAG promoter is ubiquitously active, all cells should in theory express pericam. Pericam expression was followed in the brain of double transgenics by mRNA insitu hybridization of fresh frozen sections and western blotting of whole brain extracts.

The results from insitu hybridization were not clear (data not shown). Pericam protein could not be detected by western blotting with polyclonal antibody raised against Green Fluorescent protein (results not shown). Thus, with this crossing, no expression of pericam could be observed. In order to check if the stop cassette was deleted in these mice, DNA from tail biopsies digested with EcoRV was subjected to southern blotting with the stop cassette probe. In all double positives (mice that carried both the pericam

and cre transgenes as determined by southern blotting and PCR) the stop cassette was still present. Thus it was concluded that the cre expression in this strain is not adequate for excision of stop cassette. The other possibility that the stop cassette is undeletable because of some mutation could be ruled out because the constucts were tested successfully for excision of stop cassette in *E.coli* that expresses cre (data not shown)

7.4. Breeding of pericam mice with CamKIIα-iCre mice

CamKIIα is a gene that is expressed in many cell types and tissues in the fore brain including the olfactory system. A mouse strain that expresses the cre recombinase under the CamKIIα regulatory elements was kindly provided by Dr Günther Shutz's lab in Heidelberg (Casanova et al., 2001). I crossed the pericam transgenic mice strains with the CamKIIα-Cre mice strain. Since both mice strains were heterozygous for the transgenes, it was necessary to geneotype the progeny to identify the double transgenics.

7.4.1 Ratiometric pericam is expressed in the fore-brain of double transgenics

A PCR was developed to genotype the pericam transgenic mice. The primers were directed against the GFP portion of ratiometric pericam and amplified a 300 bp region. The iCre gene in CamKIIα-iCre was also detected by a PCR developed by the group that generated the strain. Double transgenic progeny were identified by PCR and pericam expression was examined by western blotting of extracts from forebrain (where the CamKIIα-iCre is most expressed). Easily detectable expression of pericam was seen in two of the five lines tested, viz. CSRSun 54 and CSRSun 67 with antibodies directed against the calmodulin portion of ratiometric pericam at the correct molecular weight of 48 KD (Figure III.14 A). The findings were corroborated with a polyclonal antibody raised against GFP, which was also able to detect pericam (Figure III.14 B). The latter

finding is important because GFP antibody could be used in immunohistochemistry of sections from olfactory tissues (see below) Further more, it was found that pericam expression was dependent on iCre expression. No pericam expression was detected in the absence of iCre transgene in the lines that are proficient in expressing pericam. This confirms that the stop cassette is working as desired, which was in doubt in light of the observations from the experiments in which pericam mice were crossed to CMV-Cre mice. The two lines that showed significaant expression were kept for further analysis and the other lines were sacrificed.

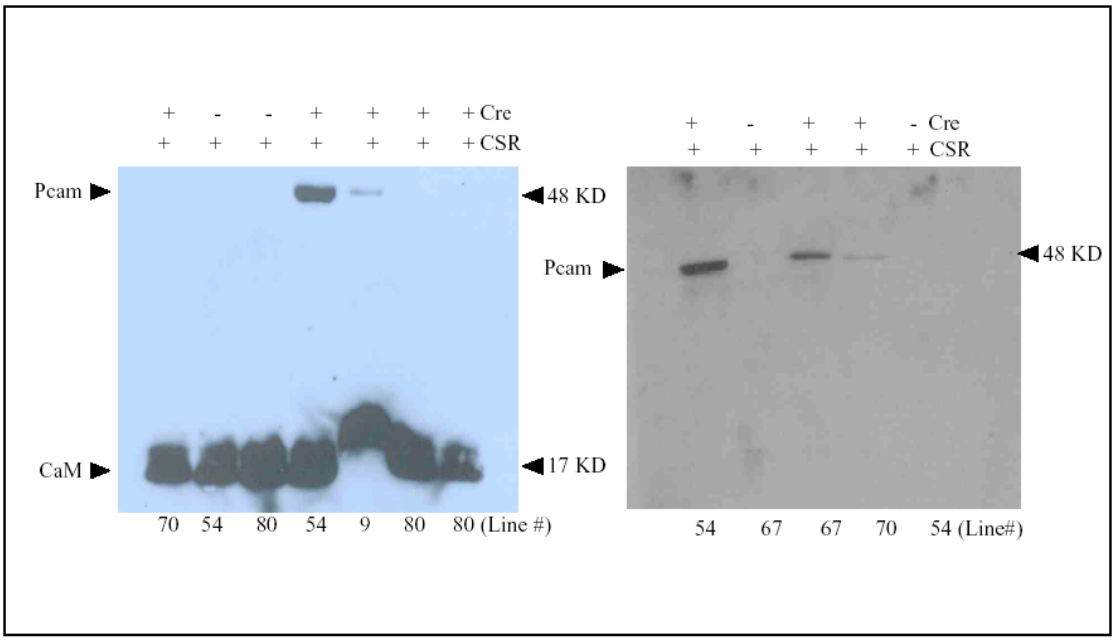


Figure III.14: Detection of pericam protein in extracts from fore brain of double-transgenics with a monoclonal anti-calmodulin antibody (A) and a polyclonal anti-GFP antibody (B). The calmodulin anti-body detects the native calmodulin at 17KD and the calmodulin portion of pericam at 48KD. Note the cre dependence of expresssion shown mainly in B. Pcam is Ratiometric pericam CaM is Calmodulin. The numbers in the bottom refer to the mouse lines.

Table III.1

	Genotype Peri/cre	**Pericam expression anti-CaM**	**Pericam expression anti-GFP**	**Result**
C**SRSun 54**	+/- +/+	Negative +++	Negative +++	Bred for further analysis
C**SRSun 67**	+/- +/+	Negative ++	Negative ++	Bred for further analysis
C**SRSun 70**	+/- +/+	Negative + (very weak)	Not tested Negative	Sacrificed
C**SRSun 9.28**	+/- +/+	Negative +(Very weak)	Negative + (Very weak)	Sacrificed
C**SRSun 80**	+/- +/+	Negative Negative	Not tested Not tested	Sacrificed

.

7.4.2 Pericam could be detected in olfactory sensory neurons and at a lower level in granule cells

In the olfactory system, CamKIIα is expressed in the olfactory sensory neurons and the granule cells in the olfactory bulb. Pericam expression was studied by immunohistochemistry using GFP antibody in cryosections of the olfactory turbinates and the olfactory bulb. Robust expression of pericam was seen in sections of the olfactory epithelium in cells that resembled olfactory sensory neurons (Figure III.15 B). Expression was not seen throughout the epithelium, but in patches in diverse locations within the epithelium. In the olfactory bulb, expression was much lower. An inner cell mass that showed a rather weak signal was readily seen (Figure III.15 A). Thus, pericam expression was both cell type specific and relatively high level, at-least in the olfactory epithelium.

These results should be seen in light of the fact that the GFP antibody used for detecting pericam is not optimal for its detection

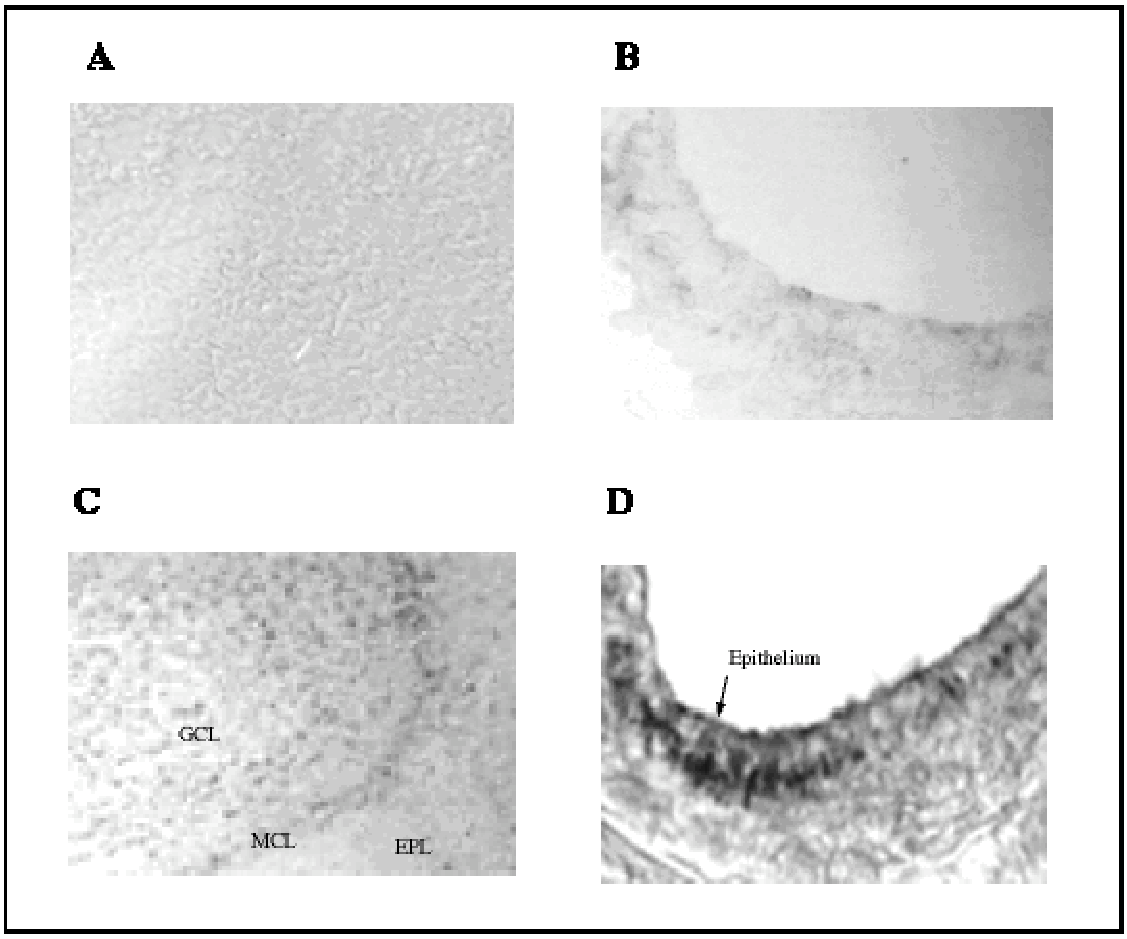


Figure III.15: Pericam expression revealed in the olfactory bulb (A and C) and the olfactory epithelium (B and D) by immunohistochemistry. Top half is from sections without primary antibody while bottom half received both antibodies. In the olfactory bulb a cell layer internal to the mitral cells is lightly labeled while in the epithelium a stronger staining in patches of the sensory neurons is seen. Magnification is 200X

7.4.3 Pericam activity could not be detected with optical imaging in the olfactory bulb

Odorant evoked activity in the olfactory bulb could include calcium transients in the granule cells, which express CamKIIα. Optical imaging was used to detect fluorescence changes associated with activity in granule cells. A window was opened up in the skull above the olfactory bulb in anesthetized animals that were then immobilized in a head holder and stimulated with odorants. Fluorescence changes were recorded with a CCD camera in both double positive and cre negative, pericam positive animals. Broadly distributed stimulus dependent changes were found throughout most part of the imaged area, but showed no specific peak in regions corresponding to granule cells that synapse with efferents from glomeruli that process an odorant, as may be expected. Responses were indistinguishable between odorants (Figure III.16). In addition, activity was also found in cre negative animals also and it was concluded that it reflects the intrinsic fluorescence changes due to blood flow changes, differences in oxygenation of hemoglobin etc and that no granule cell specific patterns could be detected. Such activity was detected as the prominent component in many intrinsic imaging studies in the olfactory bulb (Meister et al 2001).

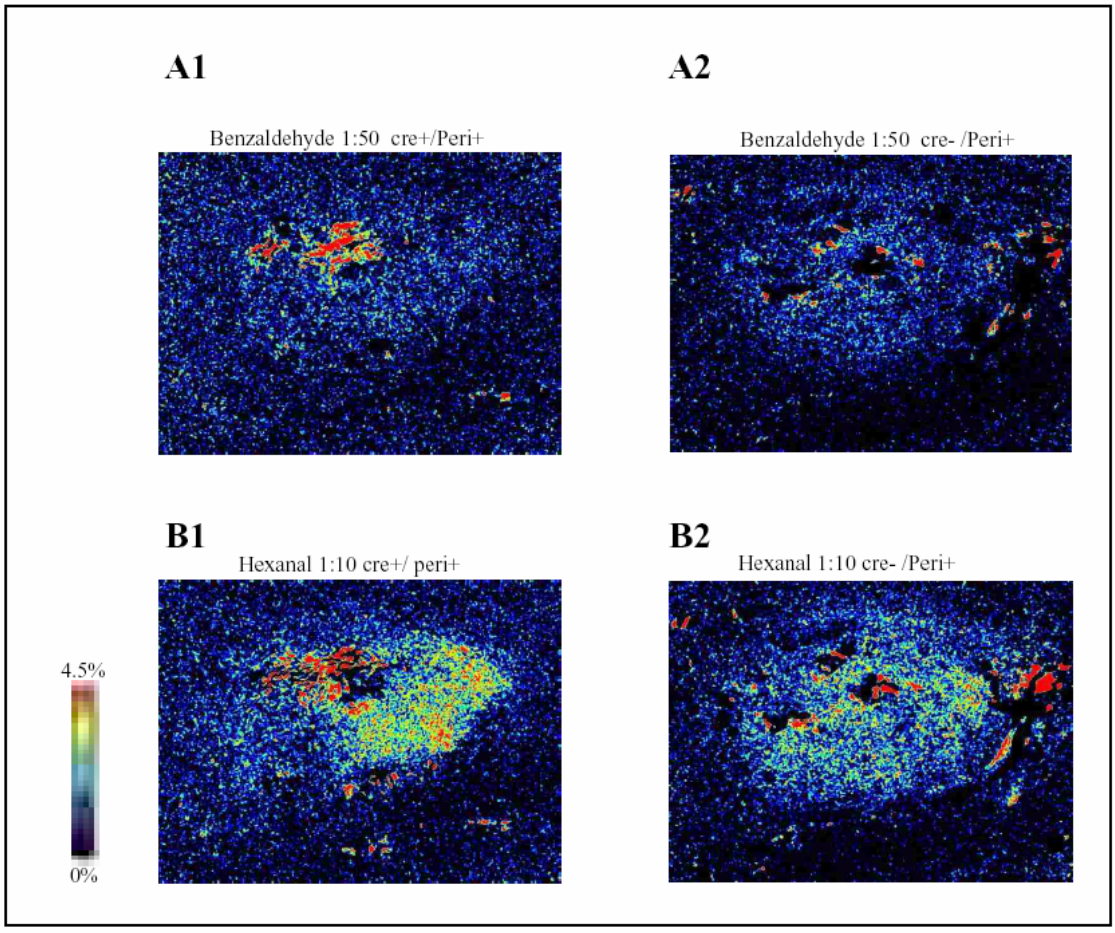


Figure III.16: Odorant evoked activity patterns in the olfactory bulb of pericam transgenic mice. Fluorescence changes in the olfactory bulb as a result of odorant stimulation was monitored optically in both pericam positive, cre positive and pericam positive, cre negative animals. Note that no difference can be seen between the two genotypes in response to relatively high concentrations of both benzaldehyde and hexanal.

IV DISCUSSION

1.Experimental strategy

The experiments outlined here aims to use fluorescent reporter genes as neuronal activity markers. Two different strategies have been followed, one that uses a Ca^{2+}- sensing protein under a ubiquitous promoter and another that uses a fast turnover fluorescent protein under an activity-dependent promoter. The activity dependence is a property of the Ca^{2+} sensing protein while in the case of the *C-fos* driven reporter; it is a property of the *C-fos* promoter.

1.1Strategies for cell type-specific gene expression in transgenic mice

There are many instances in which cell type-specific gene expression is desired. In neurobiology, it is often desired to study the activity of ensembles of neurons that specifically process a particular step in the transduction of the sensory stimulus. The mitral cells in the olfactory bulb constitute one such group. They receive information from the receptor neurons and after processing it by means of the neuronal interactions described earlier, transduce it to the olfactory cortex. As a distinct cell type, it has genes that control its fate, that are mostly transcription factors like *T-bet*. As such genes are restricted to a particular cell type, they have been harnessed to drive selective gene expression in the respective cells. Strategies have been developed whereby the transgene is directly hooked to promoters of such genes, but this is fraught with problems of inadequate expression as these promoters are often weak. To overcome this problem, amplification systems like the tetracycline responsive system and the UAS-Gal4 system have been developed. In the tetracycline system, the tet transactivator is expressed under a cell type-specific promoter and the transgene is under the control of the tet-responsive promoter. An additional level of (temporal) control is provided by the fact that transactivators and promoters have been developed that can be switched on and off by

dietary supplimentation of tet derivatives like doxycycline (Zhu et al., 2002). In the UAS-Gal4 system, the Gal4 transcription factor is expressed under the control of the cell type specific promoter and the transgene under the UAS enhancer (to which Gal4 binds) and a minimal promoter (Ornitz et al., 1991). Amplification can be achieved by including many copies of the UAS sequence and fusing the Gal4 DNA binding domain to effcient transactivators like VP16. This system is not very popular in mouse genetics. The most popular system for cell-type specific expression in mice is the cre-loxP system (Kos, 2004; Kuhn and Torres, 2002). The bacterial recombinase cre catalyses the excision (deletion) of DNA sequences between two loxP sites of same orientation. In this approach, the transgene is under the control of a suitable promoter (mostly strong and ubiquitous) but blocked by a transcriptional or translational stop cassette. Crossing to the cre strain which expresses cre under a suitable cell type specific promoter enables the expression of the transgene. Note that all these strategies necessitate the development of two different transgenic strains each.

2. *C-fos*, a widely used neuronal activity marker

Monitoring *C-fos* mRNA or protein after sensory or pharmacological stimulation has been used for more than a decade now to identify the neurons involved in a particular stimulus response pathway. *C-fos* and other Immediate-Early Genes (IEGs) are transcription factors up-regulated by neuronal excitation that may involve voltage changes, Calcium influx etc and accumulates transiently to modulate the expression of various other genes (Farivar et al., 2004). They act as the transcriptional link between short term changes in neurons upon activity and long term phenotypic changes. Stimulus dependent expression of *C-fos* has been reported from the olfactory bulb also. For the ease of monitoring *C-fos*, reporter mice strains that expresses LacZ under the control of *C-fos* regulatory elements have been constructed. In these strains, all cells in which *C-fos* become active expresses the reporter. In this study, I describe here the construction of a *C-fos* reporter strain in which only desired cell types can express the reporter.

2.1. Fos.d2EYFP mouse

In order to have a mouse strain in which only desired cell types express the reporter, we designed the *fos*-STOP-IRES-d2EYFP line. In this line, a STOP-IRES-d2EYFP cassette is inserted into a truncated *C-fos* gene. The stop cassette is a linker sequence and a polyadenylation signal that is floxed and when placed upstream of an cistron, blocks its transcription. It can be removed in select cells by crossing with a cre recombinase expressing strain. The IRES element promotes the expression of the cistron down stream of it. Perhaps the most critical element of the construct is the d2EYFP gene. While the other elements of the construct have all been tested in transgenic mice, d2EYFP is a relatively new version of EYFP that has not been used for transgenesis before. It is fusion between EYFP and the PEST sequence from mouse ornithine decarboxylase (ODC) and turns over with a half-life of two hours. While in this property it mimics *C-fos* protein, it remains to be seen if it compromises detection because only too little protein may be available at a given time after stimulation. It was chosen over EYFP because the latter has a half life of about twenty-four hours in mammalian cells and this may seriously affect the prospect of conducting repeated stimulation and imaging as the reporter left over from a previous stimulation may mask that made upon a later stimulation. It was initially not clear where to place the cassette in the *fos* gene. Leaving the *fos* coding region intact would have affected *fos* gene dosage in transgenic mice, possibly leading to abnormalities like cancer as *C-fos* is an oncogene involved in many cellular processes. In *fos*-lacZ mice that were generated, lacZ is expressed as a fusion protein with truncated versions of *C-fos*. This strategy avoids a gene dosage effect as *fos* is truncated, but the fusion proteins are localized to the nucleus because the nuclear targeting elements of *fos* are still intact in these fusions.

We did not want a nuclear localized reporter because that would compromise the possibility of being able to visualize the axonal and dendritic compartments of the responding cells. In the recently reported *fos*-lacZ mice, lacZ is fused to *fos* at the BglII site in the *fos* coding region, leaving only about fifty aminoacids of the *fos* N terminal

region. The region after this in the coding sequence is dispensable because it does not contain any sequences that regulate transcription. The only regulatory element that lies down stream of this region is the mRNA destabilizing region in the 3'UTR of the gene (Veyrune et al., 1995). So, it was decided to insert the cassette at this site and also introduce a stop codon for *C-fos*. This makes a very truncated *fos* protein of about fifty amino acids, which is expected to be unstable in the cell. The region between BglII and SalI (which is hundred bp down stream of the stop codon) of about 1.5kb is deleted from the construct. It should be noted that the deletion was not done enzymatically, but by excluding these regions from the cassette made for BAC modification, as will be described later.

2.2 Early constructs and cell culture experiments

The *fos* construct described in the previous section was arrived at after some experimentation. As described in the previous section, initial constructs were made with the stop cassette inserted in the first intron and the IRES-EYFP in the fourth exon. This construct, which had all the regulatory elements of *Fos* intact, did express the reporter upon drug stimulation in cell culture, but the stop cassette failed to prevent reporter expression when it was present. The inclusion of stop cassette in an intron probably did not support its function. Following this, other constructs were designed. The construct in which the STOP-IRES-EYFP cassette is inserted in the SalI site following the stop codon of *C-fos* was ruled out because it left the coding region of *C-fos* intact. Another problem with these constructs is that a cassette for recombination could not be excised from them Thus, both of them were ruled out in favour of the later construct that is supported by an existing transgenic mouse.

2.3. RecA mediated BAC modification and preparation for microinjection

Several approaches were considered for generating a *fos*-EYFP mouse strain. Modifying the *C-fos* genomic locus directly by targeted recombination in ES cells was ruled out because at the most two copies of the transgene could exist per cell in this case and it may not be enough to produce the required level of reporter protein, given the fact that the *C-fos* promoter is not very strong. Other attempts for making *fos* transgenic mice made use of the standard transgene approach using about 4.8kb from the *C-fos* region which contains all the known regulatory and coding elements. This approach was ruled out because it necessitates the generation and analysis of many transgenic lines to find some that expresses the transgene at the required level. In the recent years, BAC mediated transgenesis has emerged as an alternative method for making transgenic mice facilitated by powerful and easy methods for modifying bacterial artificial chromosomes based on various recombination systems in *E.coli*. Being large and of genomic origin, they have a high probability of containing all the regulatory elements for a gene 5' and 3' of the coding region and integrates just like smaller fragments (although with lower copy number) when injected into oocytes. In addition, the expression of the transgene is relatively free from position effects and thus it is necessary to analyse far fewer lines to find one with the desired expression level. Because of these advantages, it was decided to use a modified *C-fos* BAC for transgenesis. The first step in the process was to select an appropriate BAC that carries the *C-fos* gene in the middle with sufficient blocks of DNA 5' and 3' of it. Three *C-fos* carrying BACs were identified by PCR screening of pools of BAC clones provided by RZPD, Berlin. It was necessary to carry out extensive restriction mapping with rare cutting enzymes to choose the best among them for modification. The clone that was finally chosen contained a 188kb insert with 25kb 5' and 158kb 3' of the *C-fos* gene. This was considered sufficient because all the 5' regulatory sequences of *C-fos* are contained in a region about 500bp upstream of the transcription initiation site. A concern about BAC transgenesis is that it may introduce additional copies of unintended

genes to the transgenic animals and this could be a problem in cases where gene dosage is critical for proper function. It was found that the BAC chosen included the *Jun dimerization protein 2* in addition to *C-fos*. It remains to be seen if this results in any abnormalities in the transgenic animals.

The recombination method that was available in the lab at the time was the *RecA* based one developed by Nathaniel Heintz's lab in Rockefeller NewYork, USA. PCR fragments 500bp upstream of the BglII site (homology A) and another 500bp downstream of the SalI site (homology B) were linked together by means of a 15bp homology region incorporated by means of primers at the 3' end of homology A and 5' end of homology B. The common region also contained an XhoI site where the STOP-IRES-d2EYFP cassette was inserted. Thus, the recombination cassette contained the 3kb of sequences to be inserted and deletes a 1.5kb region from *C-fos* coding region. Modification starts with introduction of the recombination cassette cloned in the tet resistance carrying pSV.1*RecA* vector into *E.coli* carrying the BAC. The plasmid integrates into the BAC by homologous recombination and this event was selected for by applying tet selection at 43ºC. The only tet resistant colonies that grow at this temperature are those that have formed cointegrates because the plasmid has a temperature sensitive origin of replication. A second (intramolecular) recombination event throws out either all of the plasmid and recombination cassette or just the plasmid (both events at about equal frequency) alone and was selected for by placing the cells in medium containing fusaric acid. It was necessary to check the fidelity of the process at each step by southern blotting. Once the second recombinants were obtained, a few were checked thoroughly by PCR and southern blotting to make sure that recombination has occurred properly and that there has been no unwanted rearrangements to the BAC because of the presence of *RecA* gene in the bacteria for an extended time.

Before injection, we wished to remove the backbone of the BAC because it has loxP sites that may recombine in an undesired manner with the loxP sites in the transgene sequences. This can be done best by digestion with NotI followed by column chromatography. But in our hands, column chromatography with two different column

materials failed to efficiently separate backbone from insert. One round of oocyte microinjection was carried out with a minor second peak that eluted after the bulk of DNA and contained minimum backbone and seemed intact, but no transgenic founders could be identified. Thus, it became necessary to remove the loxP sites from the backbone and inject intact BACs.

2.4 Red-Gam mediated recombination

It is not absolutely necessary to remove the backbone of the BAC or to linearise it before microinjection. Circular molecules containing the backbone can also give rise to transgenic founders and the expression of the transgene is not compromised in these animals. This is contrary to the finding with plasmid based transgenics where even short stretches of plasmid sequences often results in silencing of the transgene (perhaps by methylation). Because we faced technical problems in generating transgenic mice with the purified insert, we decided to inject circular BACs. A precondition for this was the removal of the wild type loxP site from the backbone (it also contains a mutant loxP site) because it could participate in undesirable recombinations with the loxP sites in the transgene when crossed to a cre mouse strain. At this time, a different recombination system based on the Red-Gam system of phage λ became available in the lab. This system works with linear DNA, enabling the use of PCR fragments for modification. Further, it requires only about 40bp of homology on either side of the insert for recombination, which can be incorporated into 5' region of PCR primers which have their 3' regions homologous to a cassette (usually coding for drug resistance) that replaces the fragment to be deleted. Primers were designed with 5' regions of homology to the backbone of the BAC flanking the wild type loxP site on one end and the *SacB* gene on the other and with their 3' regions homologous to a kanamycin resistance cassette. The recombination functions are under the control of a temperature sensitive repressor and were induced by subjecting the cells to a heat shock during the preparation of competent cells. PCR fragments were electroporated and recombinant cells were selected by plating the transformants in kanamycin plates. It is necessary to verify the correctness of

recombination by PCR and southern blotting. PCR verification was done with the forward primer used for generating the recombination cassette and a primer outside but close to the region of modification. Extensive restriction mapping coupled to southern blotting also confirmed the fidelity of recombination.

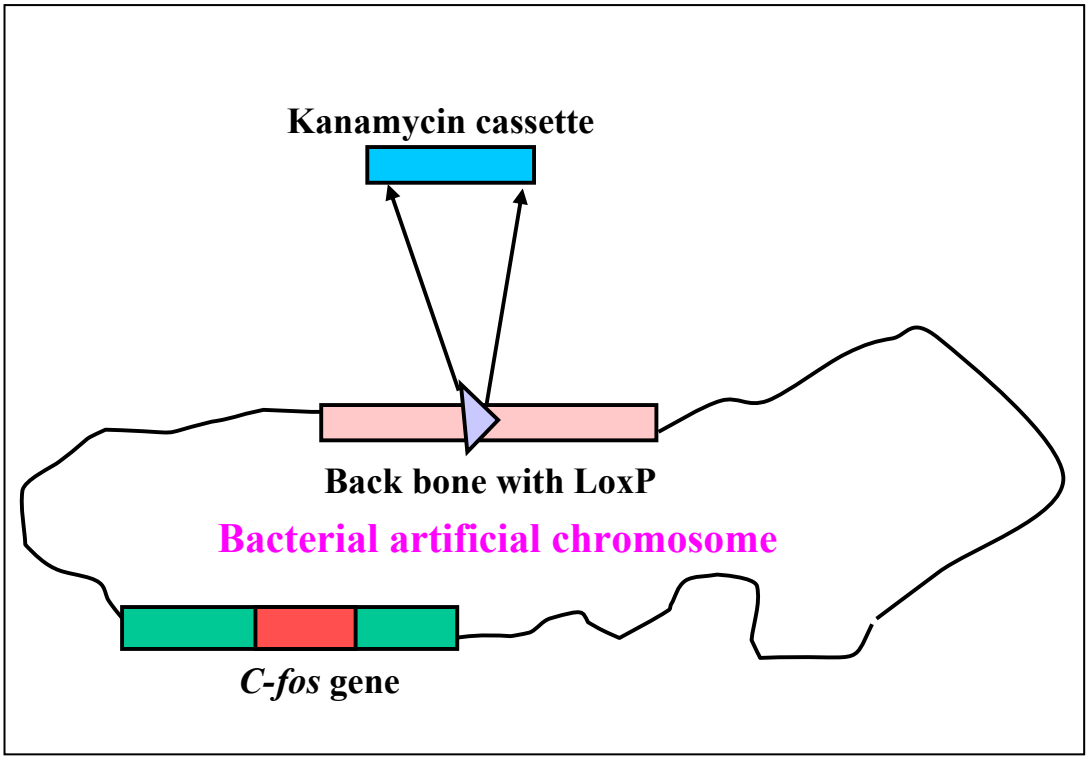


Fig 1V.1 Modifications of the *C-fos* BAC. The *C-fos* gene was modified by RecA mediated recombination to include the STOP-IRES.d2EYFP cassette and the backbone of the BAC was modified by replacement of the loxP site (triangle) with the kanamycin cassette

2.5 Microinjection and identification of transgenics

Pronuclear microinjection was carried out by our collaborator Dr Neil Smyth from the Center for Biochemistry, Medical faculty of our university. There are many conditions

suggested for BAC injection, the main difference between them being the presence of polyamines in the injection buffer. They are thought by many to be useful because they improve the integrity of the BAC molecules. In some of the injections that we carried out, polyamines were included. Three rounds of microinjection were carried out to generate the Fos d2EYFP mouse strain. The first was done with NotI digested DNA that eluted in a second peak from the sephacryl column. Although this peak was shown to be intact by Pulsed field Gel Electrophoresis, it failed to give rise to transgenic mice when injected. Probably, the DNA was degraded in the time between column run and microinjection (as do linear DNA in low concentrations). A second attempt was made with circular BACs after removal of the loxP site in the backbone and this time, only thirty-three progeny were obtained. No transgenic founders could be identified from among these perhaps because the number was too low. In a third attempt with the same DNA, one positive founder could be identified. It is known that generally, BAC transgenesis gives rise to a lesser fraction of positives compared to plasmid based transgenesis with lower transgene copy number. In the original study that demonstrated the method, the copy number was between one and three (Yang XW et al, 1997). In another study, it was between one and four (Casanova et al 2001). I could establish germline transmission for this strain. Due to time constraints, this strain could not be analyzed further.

2.6. Future experiments with fos-d2EYFP mice

The original intention was to monitor the *fos* driven transgene in mitral cells. But, since the t-bet-cre mice strain is not yet available, it is as of now not possible to do this. But definitely, this strain will have wider applicability because *C-fos* is an important activity marker and could be used for many other cell types and brain regions, depending on the availability of cre strains. One experiment that could be easily conceived of is crossing this line to one that expresses cre ubiquitously and look at patterns of activation throughout the bulb and even other brain regions upon stimulation with an odor.

Experiments in which staining for *C-fos* mRNA was done following odor stimulation identified columns of response constituted by responding glomeruli, and the mitral and granule cells connected to it. This could be visualized in experiments using this strain without the additional step of staining. Cre strains specific for certain cell types in the olfactory system like the CamkIIα (receptor neurons and granule cells) are also available. Another interesting target is the olfactory cortex, which has not been studied in the manner outlined above.

3.Pericams and other GFP based activity markers

Several GFP based activity markers have been developed over the decade since which GFP was deployed as a fluorescent reporter that for the first time enabled imaging of suitably manipulated live cells and tissues. Extensive mutagenesis and fusion to other molecules enabled development of new probes that could in real time monitor Ca^{2+} dynamics, membrane capacitance, Cl^- levels, vesicle fusion in synapses etc (Griesbeck, 2004). The field of study that has benifited most from these developments is neurobiology (Miesenbock, 2004). This section describes the development of a transgenic mouse line that can be made to express a calcium-sensing protein ratiometric pericam.

There are basically two different types of calcium sensing GFP based proteins. In dual emission sensors, two spectral variants of GFP, CFP and YFP are linked to calmodulin and a calmodulin target and calcium binding by calmodulin leads to conformational changes that brings together the two halves of the molecule making fluorescence resonance energy transfer (FRET) possible between them. Cameleon is the prototype of this kind. In single emission probes, calmodulin and often a calmodulin target are present, but the GFP fluorophore is interrupted by circular permutation or insertion and is reconstituted by the binding of calcium to calmodulin and the subsequent conformational changes. Camgaroo, G-CAMP and pericams are examples of this kind (Zemelman and

Miesenbock, 2001). By far the single emission probes have been proved to be more successful in applications in transgenic vertebrates and invertebrates (Reiff et al., 2005).

3.1 Other fluorescent activity markers in transgenic animals

Two reports of construction of GFP-activity marker based transgenic mice have been made. In one case, the synaptic vesicle fusion indicator Synapto-pHluorin was expressed under the OMP promoter, which is highly active in olfactory receptor neurons. Synapto-pHlourin is a fusion between the pH sensitive GFP variant ecliptic-pHlourin and the vesicle protein VAMP-2 and it localises to the axon termini (Yuste et al., 2000). Action potentials lead to vesicle exocytosis and synapto-pHluorin is exposed to a pH change from 5.7 inside the vesicle to 7.4 in the synaptic cleft. This results in a 20-fold fluorescence change that can be recorded optically. It was found that in this mouse strain, the glomeruli were richly labeled and it was possible to record their activity by methods established for recording activity patterns in calcium sensitive-dye labeled glomeruli. Activity patterns in the bulb in animals stimulated with various aldehydes, ketones and organic acids were recorded. Patterns of activity were distributed throughout the dorsal surface of the bulb that was imaged, but it did not change systematically with changes in carbon chain length, i.e., the chemotopy that was established with other methods was lacking in this study. The patterns also correlated with that recorded in transgenic mice in which the OSNs were also labeled a calcium sensitive dye, rhodamine dextran (Bozza et al., 2004). In another study, mice strains were generated in which the calcium sensitive proteins camgaroo-2 and inverse pericam were expressed under the bi-directional tet transactivator (tTA) responsive promoter Ptetbi. The positive founders were crossed to a mouse strain that expresses tTA under the CamKIIα promoter and many of these lines showed expression of the reporters in neurons including those of the olfactory system,which could be suppressed by adding doxycycline to the diet of the animal(Hasan

et al.2004). The authors also report successful imaging of stimulus induced fluorescence changes in the olfactory system in these mice.

3.2 Generation of Ratiometric Pericam transgenic mice

We wanted to express ratiometric pericam cell type specifically and we decided to use the stop cassette in this construct also. Among the pericams, inverse pericam has the highest affinity to calcium, but its fluorescence decreases as a result of calcium binding. Ratiometric pericam has slightly lower affinity, but can correct for concentration differences between different cells. The important issue left was which promoter to use for this strain. For high level expression of chosen transgenes in mouse, the CAG promoter has been used before. 'Green mice' that express GFP ubiquitously have been generated with this promoter and all cells excluding erythrocytes and hair are fluorescent in this strain. The promoter is a hybrid between the actin and globin promoters and Ac and CMV-IE enhancers. It has become a convention to use this promoter when strong expression in transgenic mice is desired. We also chose this promoter and cloned ratiometric pericam and the stop cassette in the proper orientation in between the promoter and the polyA signal. The construct was tested in cells in culture and was found to function as desired. The plasmid backbone was removed and the construct was microinjected into pronuclei. Two rounds of microinjection were carried out yielding seven founders as identified by genomic southerns.

3.3 Breeding and analysis; crossing to CMV-Cre mice

In order to see cre-mediated excision of stop cassette and subsequent transgene expression, the pericam positive males were bred to females homozygous for the CMV-

Cre transgene. The progeny that are positive for pericam was expected to show ubiquitous expression. They were analyzed by in-situ hybridization and western blotting. The in-situ results were difficult to interpret because of the high background levels of staining with both pericam and stop cassette antisense probes. The western results, which are more important, were very clear, no pericam expression could be detected in whole brain extracts with anti-GFP antibody. It was an open question still if this antibody can detect pericam, but an affirmative answer was found in subsequent experiments. When tail DNA was analyzed by southern blotting with STOP cassette probe to see if it is efficiently deleted, it was found that no deletion had occurred in any of the mice analyzed. Thus, it was concluded that pericam was not expressed because of a failure in excision of the stop cassette.

3.4 Breeding of pericam mice with CamKIIα-Cre strain

We decided to breed our mice to a strain that expresses cre in at least some cells of the olfactory system. Once such well investigated strain exists- the CamKIIα-iCre strain generated by Gunther Schutz's lab in Heidelberg. This is a BAC transgenic line and is expected to recapitulate the expression pattern of CamKIIα more faithfully than the strains that have been generated based on traditional transgenic approaches. In the olfactory system, CamKIIα is known to be expressed in granule cells of the bulb and the olfactory sensory neurons (Zou et al., 2002). We analyzed double transgenic mice by western blotting and immunohistochemistry with anti calmodulin antibody for western and GFP antibody for immunohistochemistry (Calmodulin antibody could not be used for IHC because native calmodulin is ubiqiutously expressed). Ratiometric pericam could be detected in the fore brain of these mice by western blotting. Preliminary immunohistochemistry detected a rather low expression in the olfactory bulb and higher if sparse expression in the olfactory epithelium. These results however, need

confirmation. When odorant evoked activity patterns in the olfactory bulb were studied by optical imaging of fluorescent signals, specific signals were not detected, probably because of the weak expression of pericam in the bulb. But the signal in the epithelium is more reliable although more immunohistochemistry experiments need to be done to study the expression pattern. On the whole, it can be concluded that this mice strain is expressing pericam and that it could be used for further experiments

.

3.5 Future experiments with pericam transgenic mice

An important question is how good the expression is in the olfactory bulb because imaging the bulb with our system did not yield any results. Confocal or two-photon microscopy could be resorted to for imaging pericam activity as these methods (especially two-photon microscopy) can yield images and sequences from deep within the olfactory bulb. However the expression level in the olfactory bulb seem to be too low. Imaging the olfactory epithelium in double transgenics could be feasible, as expression in the receptor neurons is more robust than in the bulb. If the imaging in the olfactory bulb works (perhaps with two-photon microscopes), then, the response of the granule cells could be compared to that of the receptor neurons by loading the latter with Ca^{2+} sensitive fluorescent dyes (pericam in receptor neurons does not seem to be transported to the glomeruli) and imaging in the bulb.

DISCUSSION

V. APPENDIX

STOP –IRES-d2EYFP cassette

```
   1 ataacttcgt atagcataca ttatacgaag ttatattaag ggttccggat cctcggggac
  61 accaaatatg gcgatctcgg ccttttcgtt tcttggagct gggacatgtt tgccatcgat
 121 ccatctacca ccagaacggc cgttagatct gctgccaccg ttgtttccac cgaagaaacc
 181 accgttgccg taaccaccac gacggttgtt gctaaagaag ctgccaccgc cacggccacc
 241 gttgtagccg ccgttgttgt tattgtagtt gctcatgtta tttctggcac ttcttggttt
 301 tcctcttaag tgaggaggaa cataaccatt ctcgttgttg tcgttgatgc ttaaattttg
 361 cacttgttcg ctcagttcag ccataatatg aaatgctttt cttgttgttc ttacggaata
 421 ccacttgcca cctatcacca caactaactt tttcccgttc ctccatctct tttatatttt
 481 ttttctcgag ggatctttgt gaaggaacct tacttctgtg gtgtgacata attggacaaa
 541 ctacctacag agatttaaag ctctaaggta aatataaaat ttttaagtgt ataatgtgtt
 601 aaactactga ttctaattgt ttgtgtattt tagattccaa cctatggaac tgatgaatgg
 661 gagcagtggt ggaatgcctt taatgaggaa aacctgtttt gctcagaaga aatgccatct
 721 agtgatgatg aggctactgc tgactctcaa cattctactc ctccaaaaaa gaagagaaag
 781 gtagaagacc ccaaggactt tccttcagaa ttgctaagtt ttttgagtca tgctgtgttt
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 901 tacaagaaaa ttatggaaaa atattctgta acctttataa gtaggcataa cagttataat
 961 cataacatac tgtttttttct tactccacac aggcatagag tgtctgctat taataactat
1021 gctcaaaaat tgtgtacctt tagcttttta atttgtaaag gggttaataa ggaatatttg
1081 atgtatagtg ccttgactag agatcataat cagccatacc acatttgtag aggttttact
1141 tgctttaaaa aacctcccac acctcccccct gaacctgaaa cataaaatga atgcaattgt
1201 tgttgttaac ttgtttattg cagcttataa tggttacaaa taaagcaata gcatcacaaa
1261 tttcacaaat aaagcatttt tttcactgca ttctagttgt ggtttgtcca aactcatcaa
1321 tgtatcttat catgtctgga tctgacatgg taagtaagct tgggctgcag gtcgagggac
1381 ctaataactt cgtatagcat acattatacg aagttatgcc cctctccctc ccccccccct
1441 aacgttactg gccgaagccg cttggaataa ggccggtgtg cgtttgtcta tatgttattt
1501 tccaccatat tgccgtcttt tggcaatgtg agggcccgga aacctggccc tgtcttcttg
1561 acgagcattc ctaggggtct ttcccctctc gccaaaggaa tgcaaggtct gttgaatgtc
1621 gtgaaggaag cagttcctct ggaagcttct tgaagacaaa caacgtctgt agcgacccctt
1681 tgcaggcagc ggaacccccc acctggcgac aggtgcctct gcggccaaaa gccacgtgta
1741 taagatacac ctgcaaaggc ggcacaaccc cagtgccacg ttgtgagttg gatagttgtg
1801 gaaagagtca aatggctctc ctcaagcgta ttcaacaagg ggctgaagga tgcccagaag
1861 gtaccccatt gtatgggatc tgatctgggg cctcggtgca catgctttac atgtgtttag
1921 tcgaggttaa aaaaacgtct aggcccccccg aaccacgggg acgtggtttt cctttgaaaa
1981 acacgatgat aatatggcca caggatccac cggtcgccac catggtgagc aagggcgagg
2041 agctgttcac cggggtggtg cccatcctgg tcgagctgga cggcgacgta aacggccaca
2101 agttcagcgt gtccggcgag ggcgagggcg atgccaccta cggcaagctg accctgaagt
2161 tcatctgcac caccggcaag ctgcccgtgc cctggcccac cctcgtgacc accttcggct
2221 acggcctgca gtgcttcgcc cgctaccccg accacatgaa gcagcacgac ttcttcaagt
2281 ccgccatgcc cgaaggctac gtccaggagc gcaccatctt cttcaaggac gacggcaact
2341 acaagacccg cgccgaggtg aagttcgagg gcgacaccct ggtgaaccgc atcgagctga
2401 agggcatcga cttcaaggag gacggcaaca tcctggggca caagctggag tacaactaca
2461 acagccacaa cgtctatatc atggccgaca agcagaagaa cggcatcaag gtgaacttca
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2521 agatccgcca caacatcgag gacggcagcg tgcagctcgc cgaccactac cagcagaaca
2581 cccccatcgg cgacggcccc gtgctgctgc ccgacaacca ctacctgagc taccagtccg
2641 ccctgagcaa agaccccaac gagaagcgcg atcacatggt cctgctggag ttcgtgaccg
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2761 cggaggtgga ggagcaggat gatggcacgc tgcccatgtc ttgtgcccag gagagcggga
2821 tggaccgtca ccctgcagcc tgtgcttctg ctaggatcaa tgtgtagatg cgcggc
```

Fos Homology A and B

```
   1 atacgtcgac caaagagtcg ctaactagag tttgggaggc ggcaaaccgc ggcaatcccc
  61 cctcccgggg cagcctggag cagggaggag ggaggaggga aggaagggtg ctgcgggcgg
 121 gtgtgtaagg cagtttcatt gataaaaagc gagttcattc tggagactcc ggagcagcgc
 181 ctgcgtcagc gcagacgtca gggatattta taacaaaccc cctttcgagc gagtgatgcc
 241 gaagggataa cgggaacgca gcagtaggat ggaggagaaa ggctgcgctg cggaattcaa
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 541 accaggctgt gggcctcaag gacttgcaag catccacatc tggcctccag tcctcacctc
 601 ttccagagat gtagcaaaaa caaaacaaaa caaaacaaaa aaccgcatgg agtgtgttgt
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 781 tggaattaac ctggtgctgg attgtatcta gtgcagctga ttttaacaat acctactgtg
 841 ttcctggcaa tagcgtgttc caattagaaa cgaccaatat taaactaaga aaagatagga
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 961 caatgttcat tgtcatgtta ctgatcatgc attgtcgagg tggtctgaat gttctgacat
1021 taacagtttt ccatgaaaac gtttttattg tgttttcaat ttatttatta agatggattc
1081 tcagatattt atatttttat tttatttttt tctaccctga gggtcgacga t
```

CAG STOP RmPc

```
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  61 gcccatatat ggagttccgc gttacataac ttacggtaaa tggcccgcct ggctgaccgc
 121 ccaacgaccc ccgcccattg acgtcaataa tgacgtatgt tcccatagta acgccaatag
 181 ggactttcca ttgacgtcaa tgggtggact atttacggta aactgcccac ttggcagtac
 241 atcaagtgta tcatatgcca agtacgcccc ctattgacgt caatgacggt aaatggcccg
 301 cctggcatta tgcccagtac atgaccttat gggactttcc tacttggcag tacatctacg
 361 tattagtcat cgctattacc atgggtcgag gtgagcccca cgttctgctt cactctcccc
 421 atctcccccc cctccccacc cccaattttg tatttattta ttttttaatt attttgtgca
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 721 ccggctctga ctgaccgcgt tactcccaca ggtgagcggg cgggacggcc cttctcctcc
 781 gggctgtaat tagcgcttgg tttaatgacg gctcgtttct tttctgtggc tgcgtgaaag
 841 ccttaaaggg ctccgggagg gccctttgtg cgggggggag cggctcgggg ggtgcgtgcg
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1021 ggtgccccgc ggtgcggggg ggctgcgagg ggaacaaagg ctgcgtgcgg ggtgtgtgcg
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1141 cctccccgag ttgctgagca cggcccggct tcgggtgcgg ggctccgtgc ggggcgtggc
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2161 tcttgttgtt cttacggaat accacttgcc acctatcacc acaactaact ttttcccgtt
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VII. ABBREVIATIONS

A	Adenosine
AC	Adenylate cyclase
AON	Anteroir olfactory nucleus
bp	Base pairs
cDNA	complementary DNA
DNA	Deoxy ribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DMEM	Dulbecco's modified eagle medium
EEG	Electro encephalo gram
EOG	Electro olfacto gram
EtOH	Ethanol
Fig	Figure
GABA	γ-amino butyric acid
GFP	Green Flourescent protein
GPCR	G-Protein coupled receptor
h	hour

IPTG	isopropyl β-D-1-thiogalactopyranoside
IRES	Internal ribosomal entry site
Kb	Kilobase
LacZ	β-galactosidase gene
M	Molar
MCS	Multiple cloning site
μ	Micro
n	nano
OB	Olfactory bulb
OBP	Odorant binding protein
OE	Olfactory epithelium
OCNC	Olfactory cyclic nucleotide gated channel
OR	Odorant receptor
ORN	Olfactory receptor neuron
UV	Ultraviolet

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