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I am submitting herewith a dissertation written by Cymbeline T. Culiat entitled "EVIDENCE FOR THE INVOLVEMENT OF THE β3 SUBUNIT OF THE TYPE A γ-AMINOBUTYRIC ACID RECEPTOR IN MOUSE PALATE DEVELOPMENT." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

E M Rinchik

Eugene Rinchik, Major Professor

We have read this dissertation
and recommend its acceptance:

Lisa J. Stubbs

Lane B. Russell

Debney K. Johnson

Accepted for the Council:

Leo Minkel

Vice Provost
and Dean of the Graduate School

EVIDENCE FOR THE INVOLVEMENT OF THE β3 SUBUNIT OF THE TYPE A γ-AMINOBUTYRIC ACID RECEPTOR IN MOUSE PALATE DEVELOPMENT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Cymbeline T. Culiat

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This work is dedicated to my husband, JULIO R. CULIAT, for his love, humor, unwavering support and for doing more than his fair share of housework so this dissertation can be completed. It is also for my first teachers, my parents CRISPINO TANCONGCO and CINDERELLA PRIMAVERA who taught me to love learning.

ABSTRACT

Analyses of 20 radiation-induced deletion mutations of the pink-eyed (*p*) locus in mouse chromosome 7 have identified a specific genetic interval associated with a recessive, neonatally lethal mutation resulting in isolated cleft palate. This interval extends from the distal breakpoint of the p^{83FBFo} deletion to *Gabrb3,* the gene encoding the β3 subunit of the type A γ-aminobutyric acid receptor ($GABA_A$), defining a gene(s) designated *cp1*, which controls secondary palate development. Complete concordance between alterations at the *Gabrb3* transcription unit and the clefting phenotype was found in these 20 mutations. When combined with earlier teratological studies, this concordance suggested that *Gabrb3* is *cp1* and that a particular $GABA_A$ receptor that includes the β*3* subunit may be necessary for normal palate development.

The extents of the distal breakpoints of the deletions used to localize *cp1* were further differentiated by determining whether any deleted a third $GABA_A$ receptor subunit gene encoding the γ3 subunit (*Gabrg3).* The data indicated the following gene order: *p-Gabrg3-Gabra5-Gabrb3-Znf127.* A mutant mouse line that fails to express the γ3 transcript and another that expresses neither the γ3 and α5 transcripts were identified. Mice from both mutant lines are phenotypically normal and do not exhibit any of the neurological symptoms characteristic of the rare survivors that are deleted for all three (γ3, α5, and ß3) subunits but do not have a cleft palate. These two mutant lines provide a whole-organism $GABA_A$-receptor background

that is devoid of any receptor subtypes that normally contain the γ3 and/or α5 subunits. Neither *Gabra5* nor *Gabrg3*, like *Gabrb3*, is functionally imprinted in mouse brain. The absence of an overt neurological phenotype in mice lacking the γ3 and/or α5 subunits, along with the lack of imprinting of all three $GABA_A$-receptor genes in mouse brain, suggest that *GABRG3* and *GABRA5* (and quite possibly *GABRB3*) are unlikely to contribute to Angelman syndrome in humans as previously proposed.

Analysis of mouse genomic DNA by Pulsed-Field Gel Electrophoresis (PFGE), and molecular characterization of two overlapping mouse fragments covering the *cp1* interval cloned as yeast artifical chromosomes (YAC), established a physical map around *cp1* and estimated the interval to be ~100 kb. The restriction map of rare-cutting enzymes within the interval do not show the presence of any other strong CpG islands that could indicate the presence of alternate candidate genes. A comparison of this mouse physical map with the established map in human 15q11-q13 reveals a high degree of conservation between these homologous chromosomal regions.

Based on our genetic studies, on earlier *in vitro* and *in vivo* studies showing a teratological effect of GABA and its agonists in palate development, and on our RNA analysis showing expression of the gene in isolated palate shelves during the critical stage of palate shelf elevation and fusion, we hypothesized that *cp1* is *Gabrb3* and that a deficiency in a $GABA_A$-receptor containing β3 as a subunit can cause clefting in mouse. This hypothesis was rigorously tested by a phenotype-rescue experiment. Neonatally lethal cleft palate in mice homozygous for a chromosome-7

deletion ($p^{4THO-II}$) that deletes *Gabrb3* has been corrected by the introduction of a rat *Gabrb3* cDNA transgene under the control of the human β-actin promoter. $p^{4THO-II}$ homozygotes rescued from the lethal cleft palate by the expression of the *Gabrb3* transgene were observed from two independently derived lines. The rescued deletion homozygotes are typically smaller than their littermates, but this decrease in size correlates with homozygosity for the deletion rather than with expression of the transgene. The *Gabrb3* transgene is imprinted in at least one line, such that rescue occurs only when the transgene is inherited from the mother. RNA analysis showed that the transgene is expressed equally well when passed through either germline; hence, this imprinting may be a tissue specific difference. Since the *Gabrb3* gene is not imprinted endogenously, this imprinting of the transgene is probably not related to its native function. Regardless of the imprinting of the transgene in this one line, the phenotype-rescue experiment does confirm the hypothesis that *Gabrb3* is *cpl*. Moreover, the extraordinarily conserved *Gabrb3* gene has been placed in the homologous human chromosomal region 15q11-q13, which is associated with syndromes that manifest craniofacial anomalies. Thus, mutations in the human homologue *GABRB3* may contribute to inherited or sporadic craniofacial disorders in humans.

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LIST OF ABBREVIATIONS

AS	Angelman' s Syndrome
bp	Basepair
cDNA	Complementary DNA synthesized by reverse Transcription of messenger RNA
chr	Chromosome
CL/P	Clefting of the lip with or without clefting of the secondary palate
cM	Centimorgan
CP	Isolated clefting of the secondary palate
cp1	Chromosome 7 Interval/Locus containing gene(s) for cleft palate formation
CpG	Dinucleotide of deoxycytidine 5'-monophosphate (5') and deoxyguanosine 5'-monophosphate (3'), which occurs infrequently in mammalian DNA and is digested by certain restrictions enzymes (rarecutters)
dNTPs	deoxynucleotide tri-phosphate
g	Gram
GABA	Gamma (γ) aminobutyric acid, a neurotransmitter
$GABA_A$	Type-A GABA receptor
kb	Kilobase
l	liter

mb	Megabase
ml	Milliliter (10^{-3} liter)
mM	Millimolar (10^{-3} mole/liter)
MW	Molecular weight
μg	Microgram (10^{-6} gram)
μl	Microliter (10^{-6} liter)
nt	Nucleotide
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PWS	Prader-Willi Syndrome
UV	Ultraviolet
YAC	Yeast Artificial Chromosome

I. INTRODUCTION

Developing an understanding of the mechanisms of mammalian embryonic development has progressed rapidly primarily due to the isolation and characterization of genes that govern various aspects of this process. One area that is just beginning to benefit from this approach is craniofacial development. The inherent difficulty in establishing linkage of human genetic markers to prevalent facial defects (Carey, 1989; Fraser, 1989) is compounded by the tremendous influence of environmental factors in the etiology of facial abnormalities. This multifactorial nature of the etiology of facial clefting has hindered the identification of genes directly involved in specific events of craniofacial development. When this study began, over three years ago, there was no gene unambiguously shown to play a role in the etiology of the most common facial malformation in man, facial clefting. This year the homeobox-containing gene *Msx1* (Satokata and Maas, 1994) and the gene encoding the transforming growth factor β-3, *TGFβ3* (Proetzel and Doestchman, 1994), were reported to play direct roles in mouse palatogenesis. The work described here will contribute another component to the picture--the β3 subunit of the type-A GABA receptor--hence implicating the involvement of neurotransmitters. Since the morphological and biochemical bases of palate development have been described in great detail, the identification of these genes and their subsequent functional characterization can be easily integrated into the existing biological scenario of palatogenesis.

Understanding the molecular basis of normal and abnormal processes of secondary palate development will be valuable in resolving some aspects of the complex biology of mammalian facial development and in initiating the extrapolation of mouse data to human disorders. The latter can be systematically investigated, so these genetic markers could ultimately be applied to the diagnosis of CP and pedigree analyses for calculating risk for certain individuals. Finally, the functional analysis of these newly identified CP genes and determination of the factors that can adversely modify their expression may provide the basis for designing measures for reducing the risk of generating CP in the developing fetus (Freni and Zapisek, 1991; Sperber and Machin, 1994).

Isolated Cleft Palate (CP) in Man is a Very Complex Defect

Facial clefting occurs either as cleft lip with or without cleft palate, generally designated as CL/P or as isolated clefting of the palate, CP. These are developmentally and genetically distinct abnormalities (Fogh-Andersen, 1942; Fraser, 1963; Trasler and Fraser, 1963; Fraser, 1989). Embryological studies showed that the lip forms from fusion of the ends of the medial nasal swelling and the maxillary swelling at around 7-8 weeks after conception, whereas development of the roof of the mouth (secondary palate) occurs a week or two later, from the fusion of palatal shelves above the tongue (Fraser, 1963). A genetic basis for the difference in CL/P and CP was based upon the demonstration that siblings

of patients with CL/P have increased incidence of CL/P but not CP (Fraser, 1989). Likewise, siblings of patients with CP have increased frequency of CP, but not CL/P.

The incidence of CP in the human population has been calculated to be as high as 1 in 2128 live births (Fraser, 1963; Melnick et al., 1977) to 1 in 1400 births, with little racial variation (Moore, 1991). About 80% of reported cases are non-familial (sporadic). Although a multifactorial model fits the data for this group, the etiology still is unknown (Melnick et al., 1977; Christensen and Fogh-Andersen, 1993). Twelve percent of the cases show an autosomal dominant pattern of inheritance, and the remaining 8% appear to have varied genetic etiology (eg. X-linked, autosomal recessive). CP can be manifested by itself (non-syndromic) or as part of a more complex syndrome (Fraser, 1989; Fitzpatrick et al., 1994). It is amazing that at least 106 syndromes include CP as one of the phenotypes (Salinas et al., 1982), attesting to the diversity of mechanisms that give rise to this malformation. In one report, about 40% of the CP cases observed were associated with one or more major congenital defects (Fitzpatrick et al., 1994), and half of these were clearly diagnosed as part of known syndromes.

The patterns of inheritance exhibited by CP in humans are so diverse that sifting out the genetic from the environmental component is extremely difficult. Numerous hypotheses to explain the existing data have been debated (reviewed by Fraser, 1989). The Multifactorial-Threshold Model can account for the majority of CP cases (also true for CL/P). This model proposes that CP results from a cumulative effect of

genes (each with a "minor" contribution) plus detrimental environmental conditions that exceed a threshold. The threshold is believed to be the critical time of delay beyond which the palate shelves are unable to meet and fuse (Fraser, 1989). Despite the profound influence of environmental factors in generating CP, several researchers have demonstrated the involvement of genes with major effects in generating CP (Bonner and Slavkin, 1975; Vekemans et al., 1981; Francomano et al., 1987; Karolyi et al., 1990; Moore, 1991; Gorski et al., 1992). A new twin-concordance study (the most extensive undertaken so far) has also shown that genetic factors play a substantial role in CP (Christensen and Fogh-Andersen, 1993). The *CPX* locus in the human *Xq13-q21* region has been associated with X-linked, nonsyndromic cleft palate and/or ankyglossia (tongue-tiedness) (Bjornsson et al., 1989). Attempts to isolate and identify this gene by a positional-cloning approach are under way (Moore et al., 1991; Gorski et al., 1992; Stanier et al., 1993). The most current data positions *CPX* to a ~5-cM interval between the markers *DX326* and *DXYS1X*. In the mouse, three genes have been identified in the etiology of CP: *Msx 1* (Satokata and Maas, 1994) , *TGFβ3* (Proetzel and Doestchman, 1994), and *Hoxa-2* (Gendron-Maguire et al., 1993; Riiji et al., 1993). CP generated by the disruption of these genes is inherited in a recessive manner. It remains to be seen if mutations in these genes also generate CP or at least some form of facial defect in man.

An Overview of the Developmental and Biochemical Aspects of Palatogenesis

Knowledge about normal secondary palate development consists primarily of morphological (reviewed in: Fraser, 1963; Sulik and Schoenwolf, 1985; Johnston et al., 1991) and biochemical data (reviewed in Ferguson, 1988; Greene et al., 1991). This information has also been integrated into a mechanistic model by Freni and Zapisek (1991). Palatogenesis can be summarized as follows: Under the control of *Hox* transcriptional factors, the neural crest cells migrate into the primitive oral cavity to form five pharyngeal arches (Krumlauf, 1993; Sperber and Machin, 1994). The mandibular pharyngeal arches then give rise to lateral maxillary prominences that form two lateral palatal shelves. Palate shelves begin as vertical growths descending along the sides of the tongue. The oral cavity expands, and the tongue descends, allowing room for the two palate shelves to elevate rapidly and to reorient to a horizontal position over the tongue. The intrinsic force in the elevation process is probably generated by progressive accumulation and hydration of hyaluronic acid coupled with cell division. The edges of the palatal shelves (designated the median epithelial edge or MEE) meet, and programmed cell death appears to facilitate fusion of the shelves, thereby completing the intact secondary palate. An alternative mechanism of fusion by MEE cells migrating out of the seam into the nasal and oral epithelia has also been proposed (Carrete and Ferguson, 1992). Final stages involve ossification and formation of soft palate-muscle. This series of events is essentially the

same in humans (Fraser, 1963; Sulik and Schoenwolf, 1985). Problems in tongue retraction, shelf elevation, and shelf-to-shelf contact account for most cases of cleft palate (Johnston, 1991). Salinas et al. (1982) also suggested that causes of CP include anomalies of the entire upper airway, particularly the pharynx. The severity of cleft palate ranges from the readily detectable overt cleft in the roof of the mouth to the mildest alteration in the submucous layer of the palate found towards the nasal surface (Salinas et al., 1982).

In general, palatal shelves are discernible in the mouse embryo 11 days after gestation (E11). Vertical growth of the shelves beside the tongue occur at E12-E13, and is followed by elevation and reorientation to the horizontal position around E14-E15. Fusion occurs immediately after contact of the palate shelves (E15-E16). Excellent ultrastructural data of these critical stages have been presented by Sulik and Schoenwolf (1985) and Trasler and Fraser (1963). In the developing human embryo, palate shelves are discernible around 5-6 weeks after conception, and fusion is completed by 9-10 weeks (Freni and Zapisek, 1991; Fraser, 1963)

Craniofacial development is directed by delicately balanced biochemical processes, rendering it extremely sensitive to aberrant changes in cellular levels of endogenous substances and assault by other compounds (Greene et al., 1991). The sensitivity of palate development is reflected in the long list of chemicals that disrupt the process and generate CP (reviewed by Walker and Patterson, 1974; Freni and Zapisek, 1991). The most common chemicals used to induce CP experimentally in rats and mice are: glucocorticoids, retinoic acid, GABA, diazepam, excess

retinoids; and vitamin B6 deficiencies. Although mouse studies have identified various teratogens that induce CP, demonstrating the same effect in humans has been difficult. There are a number of studies indicating association of CP with a particular teratogen. Two independent investigations in the USA (Saxen and Oakley, 1975) and Finland (Saxen and Saxen, 1975) found significant correlation between maternal intake of diazepam (valium) during the first trimester of pregnancy and facial clefting. Kallen (1986) reported that the use of anti-epileptic drugs by expectant mothers consistently caused CP in their children. Although glucocorticoids, the most potent inducers of CP in mouse have not been related to CP in man, glucocorticoid receptors are found in human palatal mesenchymal cells and data suggest that either endogenous or exogenous glucocorticoids might disrupt normal palate development by advesely affecting growth and diffferentiation of palatal mesenchymal cells (Yoneda and Pratt, 1981).

The prevailing biochemical view is that a variety of hormones, neurotransmitters, growth factors, and secondary messengers interact to control cell division, differentiation, and migration in the developing orofacial region. For example, cAMP levels are critical in the growth and differentiation of palatal tissues, and a positive correlation has been found between teratogens and mutations that induce CP and changes in palatal cAMP levels. It was hypothesized that extracellular molecules trigger signalling mechanisms in embryonic palate cells to elevate cAMP levels. Ferguson (1988) and Greene et al. (1991) discuss how various chemicals probably affect palatogenesis. Freni and Zapisek (1991) have

incorporated the biochemical and teratological observations into the developmental picture.

GABA and $GABA_A$ Receptors

The decarboxylation of L-glutamate by L-glutamate decarboxylase (GAD) yields γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in mammalian brain (Zubay, 1988). When GABA binds to its receptors ($GABA_A$ or $GABA_B$) in postsynaptic membranes, an integral Cl^- channel, which is part of the receptor complex, opens up and allows the influx of Cl^- and K^+ ions. This hyperpolarizes the cell and causes inhibition of neuronal transmission.

Although the distribution of GABA has been studied primarily in the central nervous system, there is increasing evidence for its presence in non-neuronal tissues throughout development. GABA has been found in embryonic palate mesenchyme (Wee et al., 1986), pancreatic islet cells and kidney (Katoh et al., 1994), and reproductive organs prior to the onset of puberty (Mitsushima et al., 1994). The function of GABA in these tissues is not known, but it has been hypothesized that it may be an inhibitory signal in mouse palate shelf reorientation (Wee and Zimmerman, 1983) and is probably involved in the release of hormones needed for sexual differentiation in monkeys (Mitsushima et al., 1994). It seems, then, that GABA is somehow involved in the timing of specific developmental events. The idea that neurotransmitters act like hormones in mediating

developmental processes like palatogenesis was first suggested by Zimmerman and Wee (1984, 1985). This concept will be discussed at length in the course of my study.

It is not clear whether the GABA detected in non-neuronal tissues is present due to uptake mechanisms or to synthesis of the neurotransmitter. A recent study of the activity of the GAD promoter in transgenic mice shows almost exclusive expression of GAD in neurons of the central nervous system (Katarova et al., 1994), thereby arguing for uptake rather than synthesis of GABA in peripheral tissues. GABA uptake has indeed been directly demonstrated in palate mesenchyme (Wee and Zimmerman, 1985). However, Katoh et al. (1994) showed substantial expression of GAD in pancreatic islets.

The $GABA_A$ receptors are differentiated from the $GABA_B$ receptors by (1) their sensitivity to the competitive inhibitor bicuculline; and (2) by their having multisubunit glycoprotein structure (Stephenson, 1990). In contrast, the $GABA_B$ receptor is sensitive to baclofen, and it consists of a single polypeptide. Far more molecular and biochemical studies have been done on the $GABA_A$ receptors because they are the primary receptors used in most of the neurons using GABA as an inhibitory neurotransmitter. Moreover, $GABA_A$ receptors are the site of action of an broad range of pharmacologically important drugs (reviewed by Delorey and Olsen, 1992; Burt and Kamatchi, 1991; Olsen and Tobin, 1990). A more detailed discussion of the structure and function of the $GABA_A$ receptor and how it may relate to palate development in mouse will be presented later.

Identification of the Genetic Components of CP Using Mouse Models

Embryological studies show that palatogenesis and the mechanisms that produce CP are basically similar in mouse and man (Fraser, 1963),, making the mouse a convenient model for dissecting the genetic components of this malformation (Juriloff and Harris, 1988) and for testing a variety of agents for their ability to induce CP.

Several compounds (eg., barbiturates, tranquilizers, glucocorticoids) readily induce CP in mice when administered to pregnant mice (Kalter, 1979). The induction of CP was most effective when the compound was introduced at 13-14 days of gestation, the period of palate-shelf elevation and reorientation (Sulik and Schoenwolf, 1985). Teratogen-induced CP is, therefore, usually associated with aberrant palate-shelf elevation (Katz, 1988). A delay in the reorientation of palatal shelves from a vertical to horizontal position probably allows ossification to occur before shelves meet and fuse. There is variability in the susceptibility of mouse strains to develop CP in response to a given teratogen. It is believed that in the mouse population, there is a large number of genes that predispose a strain to a particular CP-inducing agent (Fraser, 1989). Glucocorticoids are the most potent inducers of CP in mouse, and thus it is significant that a major locus linked to susceptibility to glucocorticoid-induced CP has been localized to mouse chromosome 8 (Karolyi et al., 1990). This locus has not yet been cloned. The type-II collagen gene on human chr 12 has been associated with the gene for Stickler syndrome, one of the more common

syndromes associated with CP (Francomano et al., 1987) but it is not known whether mutations in the mouse homolog generates CP.

Surprisingly, the first mouse CP genes, *Msx 1* (Ferguson, 1994; Satokata and Maas, 1994); *TGFβ3* (Proetzel and Doestchman, 1994) and *Hoxa-2* (Gendron-Maguire, 1993; Riiji et al., 1993) that were identified did not come from molecular work on any of the numerous gene mutations already described (Green, 1989), but came from "knocking out" the function of genes that were originally associated with other phenotypes or functions. The homeobox gene *Msx1* facilitates epithelial-mesenchymal interactions during craniofacial bone and tooth development. CP manifested by *Msx1*$^-$ / *Msx1*$^-$ mice is apparently due to a failure of fusion of palate shelves brought about by defects in dental development that change spatial relationships in the developing face, rather than in the palate itself. A similar relationship of defects in the developing human craniofacial region (other than palate shelves) and CP was previously suggested by Salinas et al. (1982). *TGF*β3 is a polypeptide growth factor that may play a role during palate-shelf fusion, because *TGF*β3$^-$ / *TGF*β3$^-$ knockout mice exhibit palates with different degrees of clefting (Proetzel and Doestchman, 1994). The role of this gene is still being characterized in the context of palatogenesis. In null mutants of *Hoxa-2*, presumably CP is a result of multiple skeletal defects due to abnormalities in the derivation of tissues from the cranial neural crest (Gendron-Maguire, 1993; Riiji et al., 1993).

Pink-Eyed Dilution (*p*) Deletion Complex: A Tool for Analyzing CP

Mutations at the pink-eyed dilution (*p*) locus in mouse chromosome 7 are easily distinguished by a reduction of eye, skin and coat color pigmentation. Examination of the melanosomes in mutants reveal structural abnormalities and a reduction in the level of black-brown (eumelanin) but not the yellow-red (phaeomelanin) pigment (Russell, 1949). Recently, two independent studies have led to the isolation of the *p* gene and its correlation to hypopigmentation abnormalities in mouse (Rinchik et al., 1993; Gardner et al., 1993) and man (Rinchik et al., 1993). The predicted product of the *p* gene appears to be an integral membrane transporter protein that may be mediating the transport of tyrosine, the precursor of melanin biosynthesis (Rinchik et al., 1993).

At least 100 *de novo* and induced *p* mutations have been identified (Russell and Rinchik, 1990; Lyon et al., 1992; Brilliant et al., 1994). In addition to hypopigmentation, these mutations display reproductive, developmental, behavioural and craniofacial defects. Because radiation primarily causes chromosomal deletions, these pleiotropic effects were attributed to disruptions in nearby loci around *p*. The recent isolation and characterization of the *p* gene support this view.

The first hint of the presence of CP gene(s) closely linked to *p* on mouse chromosome 7 came from the report of a neutron-irradiated mutation called p^{cp} (formerly p^{11}; Phillips, 1973). Homozygotes have CP and die soon after birth, whereas heterozygotes could not be distinguished from wildtype. A few mice survived to maturity with

unaffected or slightly affected palates and were fertile. These "escapers" produced the same frequency of CP in their progeny.

At the Biology Division of Oak Ridge National Laboratory, a similar deletion at *p*, called $p^{4THO\text{-}II}$, was recovered from the progeny of males treated with tritiated water (Culiat et al., 1993) using the specific-locus test devised by W. L. Russell (1952). We have shown that this mutation deletes a gene, designated *cp1*, causing a highly penetrant (95%), recessive, neonatally lethal form of CP, most likely identical to that in p^{cp}. The genetic and molecular analyses of $p^{4THO\text{-}II}$ leading to the identification of *cp1*, will be described here. Molecular analysis of p^{cp} was undertaken by other researchers (Brilliant, 1992; Nakatsu et al., 1993) and will also be discussed in relation to our results.

The availability of 20 overlapping *p* deletions with different breakpoints extending towards the chromosomal region where *cp1* maps allowed: a) the assignment of *cp1* into a specific interval in chr 7; and b) the establishment of a physical map based on analysis of YACS covering the region. For example, orientation and extent of coverage of YACS are readily determined by hybridization of YAC-derived probes into a panel of deletion DNAs. Access to the great resource of *p* deletions generated at the Oak Ridge National Laboratory was the key to the successful identification of the *cp1* gene. A review of the utility of deletion complexes in the mouse for functional and physical mapping of the mammalian genome has been published by Rinchik and Russell (1990).

Genetic and Molecular Approaches Towards the Identification of *cpl*

This work is composed of four major experimental phases representing different aspects of the combined genetic and molecular analyses of the $p^{4THO-II}$ mutation, and is geared towards the identification and isolation of *cpl*. The background literature, materials and methods, discussion, and presentation of the raw data pertaining to various phases of this dissertation are written separately for each section, but references cited in all discussions of this work are pooled at the end of the dissertation. The first two sections describing experimental work (II and III) are papers published in *Proceedings of the National Academy of Sciences* (Culiat et al., 1993; 1994); the third (section IV) is unpublished data; and the last (section VI) is a completed manuscript being submitted to *Science*.

The first phase of this project [**Section II: Defining the Interval containing the Cleft Palate 1 (*cpl*) Gene and Evidence for *Gabrb3* being a Strong Candidate Gene**] was directed towards the localization of *cpl* to a specific interval distal to *p* in chr 7 by genetic complementation analysis. A complete concordance between disruptions at a previously mapped gene, *Gabrb3*, and lethal CP was observed. This concordance was supplemented by gene expression studies, that showed absent or aberrant *Gabrb3* transcripts in animals with CP. At this point, we speculated that *Gabrb3* might be *cpl* (Culiat et al., 1993).

Further refinement of the map around *cpl* was achieved by mapping another gene in the area, the γ3 subunit of $GABA_A$ (*Gabrg3*)

[**Section III: Mapping *Gabrg3* to the *cp1* Interval and Identification of Mice Deleted for certain $GABA_A$ Receptor Subunit Genes**]. The phenotypes of *p* compound heterozygotes that were deleted for *Gabra5* and *Gabrg3*, or *Gabrg3* alone, were also examined.

The third phase of this work describes the construction of a physical map surrounding the *cp1* interval [**Section IV: Physical Mapping of the *cp1* Interval**]. This study was undertaken in order to estimate the distance of the area containing the gene of interest and to establish a map of clusters of rare-cutting enzyme sites or CpG islands. The latter are landmarks for the position of many mammalian genes. The physical size of the *cp1* interval and the density of CpG islands mapping within the interval will indicate the presence of other potential candidate genes for *cp1*. Genomic clones corresponding to the area of interest were isolated, so that reagents would be available for further experiments that may be necessary to search the area for alternative candidate genes.

Finally, simultaneously with the experiments in section IV. a phenotype rescue of $p^{4THO-II}$ homozygotes was conducted to test directly the hypothesis that *Gabrb3* is *cp1* [**Section V: Rescue of $p^{4THO-II}$ / $p^{4THO-II}$ Mice from Lethal Cleft Palate by Expression of a *Gabrb3* Transgene**]. The combination of genetic, molecular and teratological data supporting the hypothesis was persuasive enough to warrant a rigorous test. A genetic scheme was devised to recover $p^{4THO-II}$ / $p^{4THO-II}$ animals transgenic for a full length rat *Gabrb3* cDNA and to examine whether the expression of the gene alleviated or, at best, completely restored the normal palate.

All these data will be discussed in the light of the existing biochemical, teratological, and molecular data on palate development, and models will be proposed to incorporate these ideas. Current and future experiments to elucidate the role of *cpl/Gabrb3* in palate development will also be addressed.

II. DEFINING THE INTERVAL CONTAINING THE CLEFT PALATE 1 (*cp1*) GENE (S) AND EVIDENCE FOR *Gabrb3* BEING A STRONG CANDIDATE GENE

Introduction

The radiation-induced mutation $p^{4THO-II}$ disrupts a major locus (designated *cp1*) regulating palate development because homozygotes exhibit a recessive, highly penetrant clefting of the secondary palate. Initial molecular characterization of $p^{4THO-II}$ indicated that it is deleted for a part of the *p* gene, and that it extends distally to include *Gabrb3*, the gene that codes for the β3 subunit of the type–A γ-aminobutyric acid receptor (Nicholls et al., 1993). *cp1* must therefore be located in the chromosomal region bracketed by *p* and the distal breakpoint of $p^{4THO-II}$ in mouse chromosome 7. In order to narrow down the location of *cp1*, 19 other independently derived *p* deletions were tested for their ability to complement $p^{4THO-II}$ for the facial-clefting phenotype. The distal portions of these 19 mutations deleted overlapping portions of chromosome 7, between *p* and the distal breakpoint of $p^{4THO-II}$. Deletions that extended into the *cp1* locus will not complement $p^{4THO-II}$ for the CP defect, while deletions that do not include *cp1* will. It was therefore necessary to differentiate the extent of the distal breakpoints of the 19 *p* deletions for this analysis to be effective in localizing *cp1* to a very defined region. The

presence of the gene encoding the α5 subunit of the $GABA_A$ receptor (*Gabra5*) between *p* and *Gabrb3* allowed the distinction between distal endpoints of the *p* deletions used in the complementation analysis. Assigning the *cp1* locus to a very specific interval (eg., between the distal breakpoints of two *p* deletions) is a springboard for the application of techniques that will lead to the ultimate isolation and cloning of the *cp1* gene(s) based on its chromosomal position --- an approach called "positional cloning" or "reverse genetics" (reviewed by Collins, 1992; Stubbs et al., 1993).

This section will present the results of the genetic complementation analysis. Furthermore, it will discuss the preliminary data that support the hypothesis that *Gabrb3* is a strong candidate gene for *cp1*. The material in this section was published as an article in the journal *Proceedings of the National Academy of Sciences*, and was entitled "**Concordance Between Isolated Cleft Palate in Mice and Alterations within a Region Including the Gene Encoding the β3 Subunit of the Type-A γ-aminobutyric Acid Receptor**" (Culiat et al., 1993).

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Concordance Between Isolated Cleft Palate in Mice and Alterations within a Region Including the Gene Encoding the β3 Subunit of the type A γ-aminobutyric Acid Receptor

Cymbeline T. Culiat[§†], Lisa J. Stubbs[§], Robert D. Nicholls[#] Clyde S. Montgomery[§], Liane B Russel[§] and Eugene M. Rinchik[§]

[§]Biology Division and [†]University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8077 (U.S.A.), [#]Department of Neuroscience and Pediatrics, University of Florida College of Medicine, Gainesville, FL 32610.

Abstract

Genetic and molecular analyses of 20 radiation-induced deletion mutations including the pink-eyed dilution (*p*) locus in mouse chromosome 7 have identified a specific interval on the genetic map associated with a neonatally lethal mutation that results in isolated cleft palate (CP). This interval, closely linked and distal to *p*, and bracketed by the genes encoding the α5 and β3 subunits of the $GABA_A$ receptor (*Gabra5* and *Gabrb3*, respectively), contains a gene(s) (*cp1*; cleft palate 1) necessary for normal palate development. The *cp1* interval extends from the distal breakpoint of the prenatally lethal p^{83FBFo} deletion to *Gabrb3*, the gene encoding the β3 subunit of the type A γ-aminobutyric acid receptor. A complete concordance between alterations at the *Gabrb3* transcription unit and inability to complement the CP defect was observed. These mapping data, along with previously described *in vivo* and *in vitro* teratological effects of GABA or its agonists on palate development, suggest the possibility that a particular $GABA_A$ receptor that includes the β3 subunit may be necessary for normal palate development. Furthermore, the placement of the *cp1* gene within a defined segment of the larger *D15S12h* (*p*)-*D15S9h-1* interval in the mouse suggest that the highly homologous region of the human genome, 15q11-q13, should be evaluated for a role(s) in human fetal facial development.

Background

Abnormalities in facial development are among the most common and emotionally wrenching birth defects seen in the general population (Cohen, 1983). Dissection of the genetic component of particular facial development defects - for example, cleft lip with or without cleft palate (CL/P) in humans - has proved complex, and many hypotheses as to the mode of inheritance have been debated (Fraser, 1989). Although most cases of nonsyndromic CL/P are best accounted for by a multifactorial model, some major loci have been implicated in its etiology (Fraser, 1989; Chung et al., 1986; Ardinger et al., 1989; Chevenix-Trench et al., 1992), and there have been conflicting reports of linkage in humans between the transforming growth factor α gene (*TGFA*) and a major CL/P locus (Ardinger et al., 1989; Chevenix-Trench et al., 1992; Hecht et al., 1991). Despite the many remaining current uncertainties about the mode of inheritance of clefting defects, it is known that CL/P is genetically and developmentally distinct from isolated cleft palate (CP) (Fogh-Andersen, 1942) and that a genetic locus in the X chromosome in humans is implicated in clefting defects (Gorski et al., 1992 and the references therein).

The mouse has proved to be an exceptional model system for studying the effects of teratogenic and/or intrinsic genetic factors in palate development. Problems in palate-shelf elevation and fusion are believed to be among the primary reasons for clefting of the secondary palate (Johnston and Bronsky, 1991). Clefting defects can be induced in mice

by a wide range of compounds administered to pregnant females on days 13-14 of gestation (for review, see Kalter, 1979) and are usually associated with a delay in the reorientation of palatal shelves from a vertical to horizontal position, which allows ossification to occur before the shelves meet and fuse. Recently, a major locus associated with susceptibility to glucocorticoid-induced CP has been localized to mouse chromosome 8 (Karolyi et al., 1990).

A number of single-gene mutations in the mouse such as *Dc,* dancer; *Tw*, twirler; *oel*, open eyelids with CP; *pc*, phocomelic; *sho*, shorthead; *ur* , urogenital, also result in facial defects, including CP, as components of their phenotype (original references listed in Green, 1989). Unbalanced segregants of certain translocations also display CP in addition to other abnormalities (Cacheiro, personal communication). Phillips (1973) described a recessive, radiation-induced, neonatally lethal mutation (p^{11}, renamed p^{cp}) at the pink-eyed dilution (*p*) locus in mouse chromosome 7, that exhibits isolated CP, suggesting that a locus involved in secondary palate formation is closely linked to the *p* locus.

Here we describe the identification of another neonatally lethal mutation of *p* that manifests isolated CP and present both genetic- and molecular-mapping data that define a specific genetic region, closely linked and distal to *p*, that contains a gene or genes necessary for normal palate development. Both genetic complementation and gene expression data suggest a model in which deficiency of a particular subtype of the $GABA_A$ receptor may contribute to the clefting defects observed in mice homozygous for this mutation, consistent with earlier observations of

GABA or its agonist diazepam interfering with normal palate development in mice particularly in the stages of palate-shelf reorientation (Wee and Zimmerman, 1983).

Materials and Methods

Mice

All mice were bred at the Oak Ridge National Laboratory. The origin of additional *p* mutations is described elsewhere (Nicholls et al., 1993). Initially, complementation analyses involving $p^{4THO\text{-}II}$ and other radiation-induced *p* mutations (*p**) were accomplished by making reciprocal crosses of $p^{4THO\text{-}II}$ +/+ c^{ch} x *p** +/+ c^{ch} mice and scoring for pink-eyed-dilute progeny at birth and beyond. To collect late-gestation fetuses for gross analysis of the palate and for preparation of RNA and DNA, $p^{4THO\text{-}II}/p^{7R75M}$ females were crossed to various $p^*/p^{4THO\text{-}II}$ or *p** +/+ c^{ch} males. p^{7R75M} is a mild allele of *p* which exhibits darker pigmentation when homozygous than when heterozygous with most alleles of *p*; thus, recessive-lethal *p**s can be maintained easily in p^*/p^{7R75M} heterozygotes. $p^{4THO\text{-}II}/p^*$ fetuses at 17.5 or 18.5 days of gestation [embryonic day (E) 17.5 or E18.5] (where the morning of finding a vaginal plug was considered as day 0.5) could be classified by their pink eye color.

DNA Probes and Southern /RNA Analyses

The derivation of probe 218/219, a PCR-amplified cDNA fragment of the rat *Gabrb3* gene, is described elsewhere (Nicholls et al., 1993; Wagstaff et al., 1991). A probe (designated 300/301) for the rat Gabra5 gene was prepared by PCR of rat brain cDNA with primers 5'-GCCTTGGAAGCAGCTAAAATC and 5'-CTCAGAAGTCTTCTCCTCAGA. This PCR amplifies a 183-bp fragment extending from nucleotides 1194-1377 in the cDNA sequence (Krestchatisky et al., 1989). [This cDNA sequence was originally designated *GABRA4* in Krestchatisky et al. (1989), but it is most often designated *GABRA5* in the literature (e.g., Reis et al., 1993); thus, we shall also designate this particular gene as *GABRA5*.] Procedures for the preparation of DNA and RNA and for performing Southern- and RNA-blot analysis are described elsewhere (Nicholls et al., 1993). Poly(A)+ RNA was isolated by affinity chromatography with Mini-Ribosep oligo(dT) columns (Collaborative Biomedical Products, New Bedford, MA).

Results

CP in Mice Homozygous for a Radiation-Induced, Neonatally Lethal *p*-locus Mutation.

Intercrosses of mice heterozygous for $p^{4THO\text{-}II}$, a mutation at the pink-eyed dilution (*p*) locus that was recovered in the progeny of males exposed to tritiated water, produce pink-eyed homozygotes that usually

die shortly after birth. Because neonatal lethality, accompanied by CP, had previously been noted in mice homozygous for the presumed-radiation-induced p^{cp} mutation (Phillips, 1973), fetuses obtained from intercrosses of $p^{7R75M}/p^{4THO\text{-}II}$ mice were analyzed at E18.5 for the presence of CP. **Fig. 1 (right)** shows the CP in $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ homozygotes in contrast to a wild-type littermate that exhibits a normal palate **(Fig. 1, left)**. Because animals that are homozygously deleted for DNA at, or tightly linked to, the *p* locus do not necessarily exhibit CP (Nicholls et al., 1993; Lyon et al., 1992), the CP phenotype is likely to be caused by a mutation(s) tightly linked to *p* that is disrupted by the $p^{4THO\text{-}II}$ (and presumably p^{cp}) mutation. We have designated this locus *cp1* (cleft palate 1; designated as "CP" in Lyon et al., 1992).

Manifestation of the CP phenotype in $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ homozygotes, like that observed in p^{cp}/p^{cp} homozygotes (Phillips, 1973; Brilliant, 1992), is not completely penetrant. We did observe four exceptional pink-eyed-dilute offspring ($p^{4THO\text{-}II}/p^{4THO\text{-}II}$) in 350 progeny of a $p^{7R75M}/p^{4THO\text{-}II}$ intercross, which suggested ~95% penetrance of CP. None of the four exceptional mice had CP, although all were runty and nervous; two died at ~2.5 weeks of age, whereas the other two lived longer than 9 months, and at least one female was fertile, Tail DNA from three of these animals failed to hybridize by Southern blot analysis to the 218/219 *Gabrb3* probe, which is known to be deleted in $p^{4THO\text{-}II}$ (Nicholls et al., 1993), thereby confirming them as $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ homozygotes. Thus, the phenotypes of these exceptional,

surviving mice suggest that it is the CP disorder that is responsible for the recessive, neonatal lethality seen in homozygotes.

Deletion Mapping of the *cp1* Gene

We determined the genetic interval containing *cp1* by testing whether other radiation-induced, lethal *p* deletions can complement the $p^{4THO\text{-}II}$ CP anomaly (**Table 1**). [More detailed results of a much larger complementation analysis of *p*-locus mutations will be reported elsewhere (L.B.R., C.S.M. & E.M.R., UNPUBLISHED WORK).] Seven mutations (p^{6H}, $p^{46DFiOD}$, p^{83FBFo}, p^{80K}, p^{4R250H}, $p^{12R30Lb}$) can complement the $p^{4THO\text{-}II}$ CP defect in compound heterozygotes carrying $p^{4THO\text{-}II}$. However, 12 other mutations (p^{8R250M}, p^{47DTD}, p^{2HATh}, p^{45DTD}, p^{116G}, p^{132G}, p^{30PUb}, $p^{23DFiOD}$, p^{55PB}, $p^{7FR60Lb}$, p^{8FDFoD}, $p^{226THO\text{-}I}$) could not complement the CP phenotype. The $p^{46DFiOD}$ mutation is deleted for the *D15S12h (p)* locus as well as for the proximal marker *Myod1* but not for a probe for the *Gabrb3* locus, whereas the $p^{4THO\text{-}II}$ deletion breaks within *D15S12h (p)* and is deleted for a probe from the *Gabrb3* locus (Nicholls et al., 1993). Consequently, *cp1* must map distal to *D15S12h (p)*, within the limits of the $p^{4THO\text{-}II}$ deletion but distal to the distal breakpoint of the $p^{46DFiOD}$ deletion.

Since $p^{4THO\text{-}II}$ is deleted for the 218/219 *Gabrb3* probe, the mutations used in the complementation analysis were tested for deletion of the *Gabrb3* locus in the corresponding $p^{*}/p^{4THO\text{-}II}$ compound

heterozygote. **Fig. 2 A and B** and **Table 1** show that, except for the p^{8FDFoD} and $p^{226THO-I}$ mutations, deletion of a 9.0-kb *Pvu*II fragment detected by the 218/219 *Gabrb3* probe completely correlates with the inability of a mutation to complement $p^{4THO-II}$ for the CP phenotype.

p^{8FDFoD} and $p^{226THO-I}$ could not complement $p^{4THO-II}$ but neither was deleted for the *Gabrb3* probe used (**Fig. 2B and Table 1**); hence, we investigated whether the wild-type *Gabrb3* transcript was expressed in these exceptional combinations. **Fig. 3A** shows an RNA blot of poly(A)$^+$ RNA prepared from heads of late-gestation pink-eyed fetuses (i.e., $p^{8FDFoD/p4THO-II}$ and $p^{226THO-I/p4THO-II}$) that exhibited CP. Hybridization of this blot with 218/219 detected a size-altered, slightly larger *Gabrb3* transcript (~6 kb) (lane b; see arrow) in $p^{8FDFoD/p4THO-II}$ RNA, and no transcript at all in $p^{226THO-I/p4THO-II}$ RNA (lane e), as compared to the 5.5-kb transcript detected in RNA from wild-type littermate controls (e.g., lane c). The size-altered transcript from p^{8FDFoD} was also notably reduced in intensity when compared to the wild-type transcript found in $p^{7R75M/p8FDFoD}$ heterozygotes (**Fig. 3A**, lane a). Thus, aberrant transcription from the *Gabrb3* locus is displayed by these two mutations, even though neither deletes the 3' *Gabrb3* probe. Four of the mutations (p^{83FBFo}, p^{6H}, $p^{46DFiOD}$, and p^{80K}) that are not deleted for the *Gabrb3* probe and that are able to complement the *cpl* defect expressed the wild-type 5.5 kb *Gabrb3* transcript (**Fig. 3A**; lanes f-i). In summary, among the 20 mutations that were analyzed, there is complete concordance between genomic and/or transcriptional alterations at the *Gabrb3* locus and the inability to complement for the CP phenotype.

Because of the complete concordance between alterations at the *Gabrb3* locus and the CP phenotype, it was of interest to test whether the expression of the gene is consistent with a potential role in the development of fetal palate. Hybridization of the 218/219 probe to a Northern blot of total RNAs derived from E14.5. E15.5, or E16.5 whole fetuses, or from E16.5 fetal heads, showed that the 5.5-kb *Gabrb3* transcript is expressed at all these stages (**Fig. 3A**; lanes j-m).

Deletion Mapping of *Gabra5* Excludes its Candidancy for *cp1*

Since the gene encoding the α5 subunit polypeptide (*GABRA5*) of the $GABA_A$ receptor is linked to the *GABRB3* locus in the human chromosome region 15q11-q13 (Knoll et al., 1993), we tested whether a probe (designated 300/301), derived from the 3' segment of the published *GABRA5* cDNA (Krestchatisky et al., 1989), was likewise linked to *Gabrb3* in the mouse. **Fig. 2C** demonstrates that probe 300/301 hybridizes to a 4.0-kb *Pvu*II fragment in wild-type DNAs that is deleted in $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ DNA. All of the DNAs from mutations that affected the *Gabrb3* gene (13 of 13, including p^{8FDFoD} and $p^{226THO\text{-}I}$) are deleted for *Gabrb5* (not all data are shown, but are summarized in **Table 1**). Of the seven mutations that complement *cp1* and are not mutant at *Gabrb3*, only p^{83FBFo} is deleted for the 300/301 *Gabra5* probe (**Fig. 2C and Table 1**). These data suggest that *Gabra5* is located between *D15S12h (p)* and

Gabrb3, and that the p^{83FBFo} distal breakpoint maps between *Gabra5* and *Gabrb3*.

Integration of these complementation and molecular data thus defines a specific genetic interval containing the *cp1* locus (**Fig. 4**). This interval begins on the proximal side at the distal breakpoint of p^{83FBFo}, the mutation that deletes *Gabra5* but complements $p^{4THO-II}$ for CP. The *cp1* interval can extend distally only to the *Gabrb3* locus itself because p^{8FDFoD} and $p^{226THO-I}$ both fail to complement the CP abnormality but do not remove the entire *Gabrb3* locus.

Discussion

The results of complementation analyses among a number of lethal *p*-locus mutations, combined with molecular-mapping data, have suggested that a locus (*cp1*), responsible for recessive-lethal CP in the mouse, maps distal to *p* within a specific interval bracketed proximally by the p^{83FBFo} distal breakpoint and distally by the *Gabrb3* gene. CP is one of the abnormalities observed in offspring of pregnant mice treated with the GABA agonist diazepam drug at around E14, the critical period for palatal-shelf reorientation (Miller and Becker, 1975). Likewise, treatment of developing fetal palates in culture with diazepam, or with GABA itself, interferes with normal palate development by inhibiting palate-shelf reorientation (Wee and Zimmerman, 1983). The presence of GABA has also been reported (Wee et al., 1986) in E13-E15 fetal palate, consistent

with the expression of GABA receptors during the critical period for palate formation. In agreement with these earlier studies, we have shown by RNA-blot analysis that *Gabrb3* is expressed in whole fetuses and in fetal heads at times coinciding with the critical stages of palate development. It has not yet been determined, however, how much of this is due exclusively to the contribution of the developing fetal brain. Laurie et al. (1992) reported "very weak or no" *in situ* hybridization of a *Gabrb3*-specific antisense probe to sections of E14 rat brains; however, they did report weak signal from this probe when it was hybridized to sections of E17 rat brains. Thus, it will be necessary to assay by sensitive techniques whether *Gabrb3* expression in mouse brain at E14.5 accounts for some of all of the hybridization observed in our analysis of entire E14.5 fetus. *In situ* hybridization of *Gabrb3*-specific probes to sections of palate shelves should help resolve this question. Nonetheless, several lines of evidence (genetic mapping, expression during critical developmental times, and previous pharmacological/toxicological studies) suggest that *Gabrb3* itself may be a candidate for *cp1*, and that the lack of a specific $GABA_A$ receptor subtype, in which β3 comprises one of the subunits, may be responsible for the defects in palate formation seen in $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ homozygotes.

The $GABA_A$ receptors belong to a heterogeneous group of ligand-gated ion (chloride) channels that are composed of a number of subunits (e.g. α1–α6, β1–β3, γ1–γ3, δ, ε) (Olsen and Tobin, 1990). The genes encoding each subunit are expressed in different regions of the nervous system and during different stages of development (Laurie et al., 1992).

Structurally and functionally different receptors may be formed by certain combinations of these subunits; for example, at least five $GABA_A$ receptor subunits, each expressed in different regions of the nervous system, have been observed by immunofluorescence (Fritschy et al., 1992). Moreover, it is known that $GABA_A$ receptor RNA is expressed in tissues outside the central nervous system (Laurie et al., 1992). Thus, perhaps one of the many possible $GABA_A$ receptor subtypes might function in normal palate development, another question that may be resolved by *in situ* hybridization experiments that use probes specific for each of the $GABA_A$ receptor subunits.

It has been proposed that GABA may function *in vivo* by acting as a normal inhibitor of palate-shelf movement (Wee and Zimmerman, 1983). However, if the β3 subunit of $GABA_A$ receptor is, indeed, necessary for normal palate development, it is presently difficult to explain why the phenotype observed when a receptor subunit is deleted, as in the *p*-deletion homozygotes or compound heterozygotes reported here, is similar to that observed when an inhibitory GABAergic pathway is stimulated (Wee and Zimmerman, 1983). Perhaps any alteration, either positive or negative, in a GABAergic pathway in the palate could disrupt its normal development. In addition, if the lack of a specific $GABA_A$ subtype is indeed causally associated with defects in palate development, that subtype cannot include the α5 subunit, because the p^{83FBFo} mutation, which deletes at least a portion of the *Gabra5* gene, can complement the *cp1* defect.

The deletion of *GABRB3* in a family segregating Angelman syndrome (AS) has evoked the proposal that this gene may be involved in the pathogenesis of AS (Saitoh et al., 1992). However, this human 15q11-q13 deletion is described as being > 650 kb in a size with a large coding potential (Kuwano et al., 1992), and recently a probe for *GABRB3* was shown to map distal to the AS critical region (Reis et al., 1993). Indeed, the strong correlation between the *cpl* defect and alterations at the *Gabrb3* locus reported here, along with the finding that *Gabrb3* is not parentally imprinted in mouse brain and that mice hemizygous for this gene are phenotypically normal (Nicholls et al., 1993; Cattanach et al., 1992), argues that alternative hypotheses be considered for the role of the β3 subunit of the $GABA_A$ receptor in human development. Among these hypotheses is one wherein the β3 subunit is involved in aspects of fetal facial development. This particular hypothesis can be tested rigorously in the mouse by gene-transfer/mutant-phenotype-rescue experiments. It must be noted that the region between the distal breakpoint of p^{83FBFo} and *Gabrb3* (i.e., the current, most accurate definition of the *cpl* region) is still of unknown physical size. Thus, it is possible that the *cpl* gene maps within this region, proximal to *Gabrb3*. Nonetheless, regardless of the actual identity of the *cpl* gene itself, the strong conservation of fine-structure synteny (Nicholls et al., 1993) between the *D15S12h (p)-D15S9h-1* region in mice and the 15q11-q13 region in humans suggests that 15q11-q13 might be evaluated in the context of the genetics of facial dysmorphisms in human populations.

SECTION II.

TABLES

Table 1: Genetic and Molecular Characteristics of Radiation-induced *p*-locus Mutations used to Define the *cp1* Locus.

	$p^l/p^{4THO\text{-}II}$		Presence of: ≠		*Gabrb3*
Mutation (p^l)*	Lethality↑	CP	*Gabra5*	*Gabrb3*	mRNA∅
$p^{4THO\text{-}II}$	NL	+	—	—	—
$p^{7FR60Lb}$	NL	+	—	—	ND
p^{6H}	V	—	+	+	+
$p^{46DFiOD}$	V	—	+	+	+
p^{83FBFo}	V	—	—	+	+
p^{80K}	V	—	+	+	+
p^{25DVT}	V	—	+	+	ND
p^{4R250H}	V	—	+	+	ND
$p^{12R30Lb}$	V	—	+	+	ND
p^{8R250M}	NL	+	—	—	ND
p^{47DTD}	NL	+	—	—	ND
p^{2HATh}	NL	+	—	—	ND
p^{45DTD}	NL	+	—	—	ND
p^{116G}	NL	+	—	—	ND
p^{132G}	NL	+	—	—	ND
p^{30PUb}	NL	+	—	—	ND
$p^{23DFiOD}$	NL	+	—	—	ND
p^{55PB}	NL	+	—	—	ND
p^{8FDFoD}	NL	+	—	+	AB
$p^{226THO\text{-}I}$	NL	+	—	+	—

* Mice homozygous for $p^{4THO\text{-}II}$ and $p^{7FR60Lb}$ die neonatally; p^{6H} homozygotes die at variable times during juvenile life; mice homozygous for all other mutations die before birth.

↑ NL, neonatally lethal; V, viable

≠ Determined by Southern blot analysis with the 300/301 probe (*Gabra5*) or the 218/219 probe (*Gabrb3*). —, deletion of probe sequence; +, presence of probe sequence.

∅ Determined by Northern blot analysis of fetal head RNA. (-), no transcript detected; (+), normal amount and size of transcript detected; AB, aberrantly sized transcript detected; ND, not done.

SECTION II.

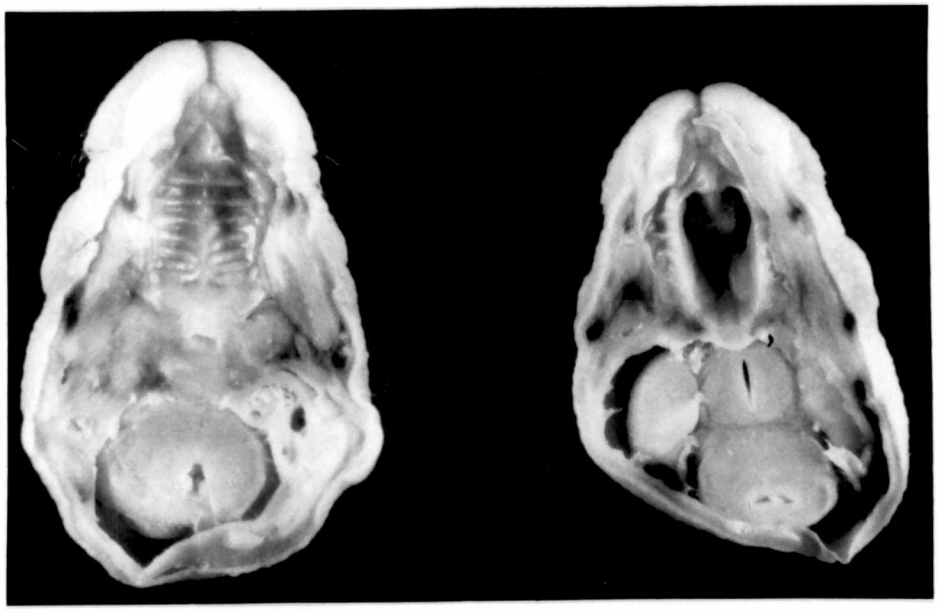
FIGURES

FIGURE 1. Secondary Palates of E17.5 Wild-type and *p*$^{4THO-II}$/*p*$^{4THO-II}$ Mouse Fetuses and Initial Analysis of the *p*$^{4THO-II}$ Mutation

Upper panel (**A**) : The ventral aspects of fixed heads, with lower jaws removed, show the normal completely fused palate in a wild-type mouse (left) and the cleft palate in *p*$^{4THO-II}$/*p*$^{4THO-II}$ homozygotes (right). It was observed that the extent of the cleft appear the same in all of the mutant fetal heads examined at this stage.

Lower Panel (**B**): Chromosome 7 map showing the initial localization of the *cp1* gene(s). The *p*$^{4THO-II}$ deletion breaks within the *p* gene and extends distally past the *Gabrb3* gene. Since the *p*^{46DFiOD} mutation, which is deleted for both *p* and *Myod1*, complements *p*$^{4THO-II}$ for the clefting defect, *cp1* must map distal to the distal breakpoint of *p*^{46DFiOD}.

A



B

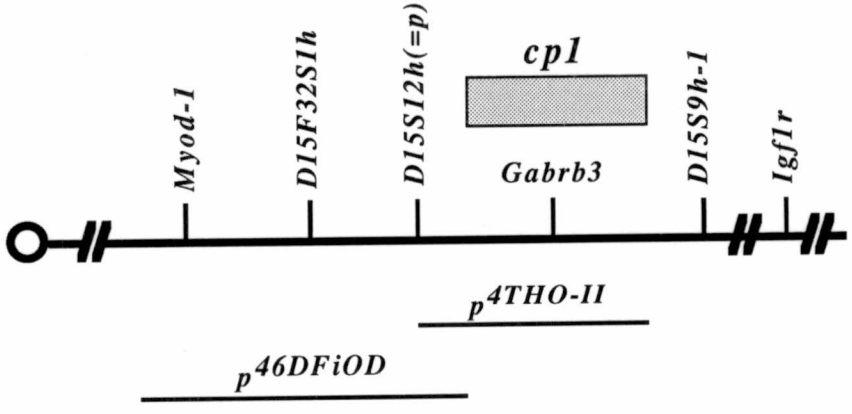

Myod-1
D15F32S1h
D15S12h(=p)
cp1
Gabrb3
D15S9h-1
Igf1r
p4THO-II
p46DFiOD

FIGURE 2. Mapping of *Gabrb3* and *Gabra5* with 20 Lethal *p* Deletions.

(A) A Southern blot of 10 μg of *Pvu*II-digested DNA, prepared from animals of the indicated genotypes, was hybridized to probe 218/219 from the *Gabrb3* locus (the 9.0 kb fragment), along with probe 34-1-111, from the *D15S9h-1* locus (Nicholls et al., 1993) as a control probe (the 4.1 and 0.7 fragments). (B-D) Southern blots of 10 μg of *Pvu*II-digested DNA, prepared from animals of the indicated genotypes, was sequentially hybridized to 218/219 (B); to *Gabra5* probe 300/301 (C); and to control probe 34-1-111 (D). The faintly hybridizing fragments observed in C are probably due to cross hybridization with related genes encoding other α subunits. Both the 4.1-kb and 0.7-kb *Pvu*II fragments detected by the control probe in D were present, but only the smaller fragment is shown.

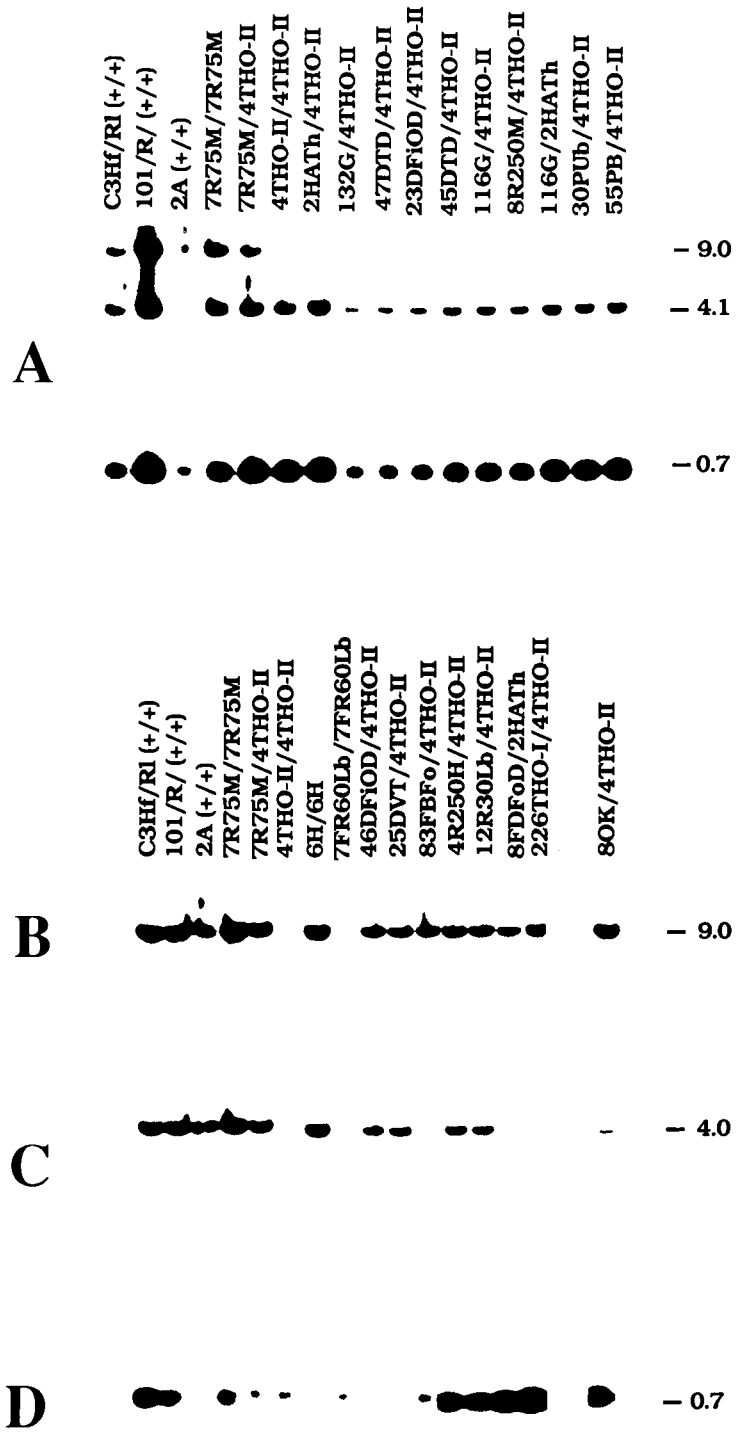

A
C3Hf/Rl (+/+)
101/R/ (+/+)
2A (+/+)
7R75M/7R75M
7R75M/4THO-II
4THO-II/4THO-II
2HATh/4THO-II
132G/4THO-II
47DTD/4THO-II
23DFiOD/4THO-II
45DTD/4THO-II
116G/4THO-II
8R250M/4THO-II
116G/2HATh
30PUb/4THO-II
55PB/4THO-II
– 9.0
– 4.1
– 0.7
B
C3Hf/Rl (+/+)
101/R/ (+/+)
2A (+/+)
7R75M/7R75M
7R75M/4THO-II
4THO-II/4THO-II
6H/6H
7FR60Lb/7FR60Lb
46DFiOD/4THO-II
25DVT/4THO-II
83FBFo/4THO-II
4R250H/4THO-II
12R30Lb/4THO-II
8FDFoD/2HATh
226THO-I/4THO-II
8OK/4THO-II
– 9.0
C
– 4.0
D
– 0.7

FIGURE 3. Expression of the *Gabrb3* Gene in Some Compound *p* Deletion Heterozygotes and in Wild-type Embryos During Stages Critical in Palate Development.

(**A**) RNA blots of poly (A)$^+$ RNA from fetal tissue were hybridized with *Gabrb3* probe 218/219. Lanes: **a-i**, E17.5 or E18.5 fetal heads: **a**, p^{7R75M}/p^{8FDFoD}; **b**, $p^{8FDFoD}/p^{4THO-II}$; **c**, +/ p^{7R75M} or +/$p^{4THO-II}$; **d**, $p^{7R75M}/p^{226THO-II}$; **e**, $p^{226THO-1}/p^{4THO-II}$; **f**, $p^{83FBFo}/p^{4THO-II}$; **g**, $p^{6H}/p^{4THO-II}$; **h**, $p^{46DFiOD}/p^{4THO-II}$; **i**, $p^{8OK}/p^{4THO-II}$; **j**, E14.5 whole fetus; **k**, E15.5 whole fetus; **l**, E16.5 whole fetus; **m**, E16.5 head. Lanes **j-m** contain RNA isolated from wild-type fetuses of the C3Hf/Rl inbred strain. The sizes of the transcripts were determined by comparison to RNA ladder from Life Technologies. The asterisk and arrow identify the 6.0-kb size-altered transcript detected by the *Gabrb3* probe from the mutant p^{8FDFoD} chromosome. In lane **c**, the genotype of the wild-type fetuses could not be precisely determined (they were either +/ p^{7R75M} or +/$p^{4THO-II}$). Lane **i** was extremely underloaded, and the transcript can be detected only after a 1-week exposure of film. (**B**) Hybridization of the same blots with a probe for chicken tubulin, a control for the amount of RNA loaded.

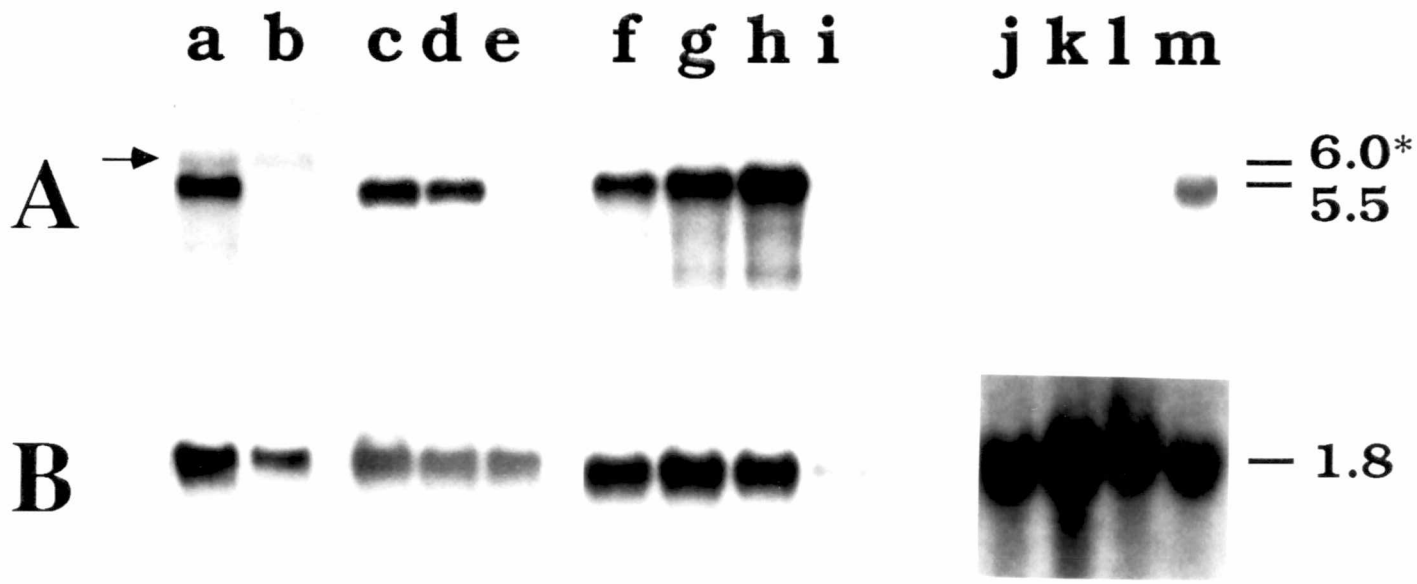
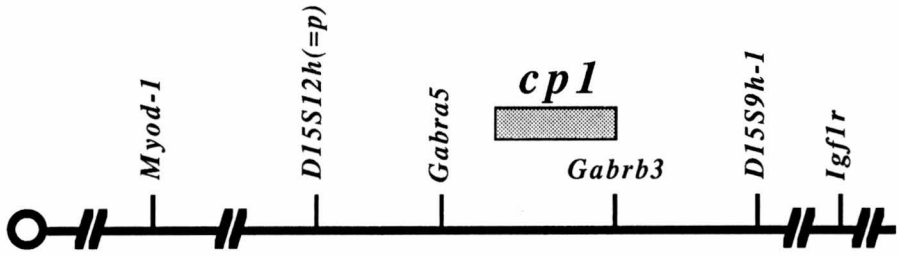

a b c d e f g h i j k l m
A
B
6.0*
5.5
1.8

FIGURE 4. Genetic Map Around the *Gabrb3* Locus

A refined genetic map surrounding the *Gabrb3* locus, indicating localization of the *cp1* gene(s). A map of the *Myod1-Igf1r* region of chromosome 7 (Nicholls et al., 1993), integrated with data reported here. The centromere is represented by the circle at the left. Extents of *p* deletions are indicated by lines below the chromosome map, and the name of each deletion is indicated. The endpoints of independently derived deletions that cannot yet be distinguished by molecular probes are shown as ending at the same place. The interval containing *cp1* is indicated by the shaded box and is bounded on the distal side by the *Gabrb3* locus and on the proximal side by the p^{83FBFo} distal breakpoint. No correlation with any physical distance is implied by spacing of loci. Recombination frequencies are as follows *Myod1*–5.5 ± 1.7–*D15S12h(p)*–1.1 ± 0.8–*D15S9h-1*–6.0 ± 1.8–*Igf1r* (Nicholls et al., 1993).



4THO-II

46DFiOD

7FR60Lb

83FBFo

8FDFoD

226THO-I

8OK

6H

25DVT

4R250H

12R30Lb

8R250M, 47DTD, 2HATh

45DTD, 116G, 132G

30PUb, 23DFiOD, 55PB

III. MAPPING *Gabrg3* TO THE *cp1* INTERVAL AND IDENTIFICATION OF MOUSE LINES DELETED FOR CERTAIN $GABA_A$ RECEPTOR SUBUNIT GENES

Introduction

Maps of highly homologous regions in different species have been instrumental in increasing dramatically the rate at which the location of genes have been determined (Nadeau, 1989). Comparative mapping between the mouse and human genomes have shown that $GABA_A$ receptor subunit genes are organized into highly conserved gene clusters. The mapping of the *cp1* locus into the chromosomal region where *Gabra5-Gabrb3* genes are located led to the idea that another $GABA_A$ receptor subunit gene might be located in this area and would therefore aid in the refinement of the map. Since there are already two α-β-γ subunit gene clusters identified in the human genome [*GABRG1*, *GABRA2*, and *GABRB1* (Schantz-Wilcox et al., 1992); *GABRG2*, *GABRA1*, *GABRA6* and *GABRB2* (Schantz-Wilcox, 1992; Hicks et al., 1994 and Russek et al., 1994)], it was highly probable that a gene encoding a γ subunit of the $GABA_A$ receptor maps with *Gabra5* and *Gabrb3*, distal to *p* in mouse chromosome 7. *Gabrg3* was the only gene encoding a γ subunit polypeptide whose chromosomal position was not known when this dissertation was first undertaken; hence, the panel of *p* deletions used in

the genetic analysis of the *cp1* locus was tested for the presence of this gene. The results showed that *Gabrg3* maps proximal to *Gabra5* allowing further refinement of the map around *cp1*.

Several distal breakpoints of the *p* deletions used in this study were further differentiated based on whether they extended into the *Gabrg3* gene. It was apparent from the additional data that certain compound heterozygotes were deleted for *Gabrg3* and/or *Gabra5*. Examination of the phenotypes of these mice was important for two reasons: (1) First, a previous report suggested that the deletion of these genes could account for the neurological traits observed in the rare "escapers" of the p^{cp} (hence $p^{4THO\text{-}II}$) mutation (Nakatsu et al., 1993). It was further hypothesized that animals deleted for these subunit genes can serve as models for the neurological human abnormality, Angelman syndrome (AS). A comparison of the characteristics of AS and the phenotypes of these *Gabrg3*$^{-}$ and *Gabrg3*$^{-}$ *Gabra5*$^{-}$ mice could address this issue. 2) Several GABA receptor subunit genes have been identified but it is not known what functions are unique for each of the different subunits. The phenotypes of mice deleted for just *Gabrg3* or a combination of *Gabra5* and *Gabrg3* might reveal the functions of these particular subunits.

The material in this section is an expanded version of a paper entitled "**Phenotypic Consequences of Deletion of the γ3, α5, and β3 Subunit of the Type A γ-aminobutyric Acid Receptor**", that was published in *Proceedings of the National Academy of Sciences* (Culiat et al., 1994).

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Phenotypic Consequences of Deletion of the γ3, α5, or β3 Subunit of the Type A γ-Aminobutyric Acid Receptor in Mice

Cymbeline T. Culiat[§†], Lisa J. Stubbs[§], Clyde S. Montgomery[§], Liane B Russel[§] and Eugene M. Rinchik[§]

[§]Biology Division and [†]University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8077 (U.S.A.),

Offprint requests to : E. M. Rinchik. Present address:

Sarah Lawrence College
1 Mead Way
Bronxville, NY 10708

Abstract

A cluster of genes encoding the α5, β3 and γ3 subunit polypeptides of the $GABA_A$ receptor (*Gabra5*, *Gabrb3*, and *Gabrg3*, respectively) was mapped distal to the pink-eyed dilution locus (*p*) on mouse chromosome 7, in the order: centromere-*p(D15S12h)-Gabrg3-Gabra5-Gabrb3*-telomere. This region shares homology with a segment of human chromosome 15 that is implicated in Angelman syndrome, an inherited neurological disorder associated with imprinting effects. We therefore tested whether these receptor subunit genes are differentially expressed depending on parental origin. None of these genes is functionally imprinted in adult mouse brain. Mice deleted for the entire subunit cluster die at birth from cleft palate. There are rare survivors that do not have cleft palate but do manifest neurological anomalies characterized by tremor, jerky gait, and runtiness. The clefting defect and the neonatal lethality has been attributed to the disruption of *Gabrb3*. In this paper we report that compound heterozygotes which are completely deleted for *Gabrg3* ($p^{46DFiOD}/p^{4THO-II}$) and, even more surprising, deleted for both *Gabrg3* and *Gabra5* ($p^{83FBFo}/p^{4THO-II}$) have no readily detectable morphological and behavioural abnormalities. These mice can now provide whole-organism $GABA_A$ receptor background that is devoid of any receptor subtypes containing the γ3 and/or α5 subunits. Since these mice do not show any readily detectable neurological phenotypes, mutations in

Gabra5 and *Gabrg3* are not useful models for understanding Angelman syndrome.

Background

In adult vertebrate brain, binding of the neurotransmitter γ–aminobutyric acid to type A ($GABA_A$) receptors results in the opening of the receptor's integral membrane Cl^- channel, which leads to an inhibition of neuronal activity (Olsen and Tobin, 1990; Schofiled et al., 1987). Other studies have suggested that during early postnatal development this receptor-ligand interaction has an excitatory function (Cherubini et al. 1991). The clinical importance of understanding the biochemistry and the regulation of expression of $GABA_A$ receptors is realized when one considers that these receptors are also targets for a wide variety of pharmaceutically important drugs, such as anti-epileptic agents, anxiolytics, muscle relaxants, sedatives and hypnotics (Schofield et al. 1987; Burt and Kamatchi 1991).

Comparison of amino acid sequences predicted from cloned cDNAs reveals that $GABA_A$ receptor belongs to a superfamily of ligand-gated ion-channels, which includes the glycine and nicotinic acetylcholine receptors (Olsen and Tobin, 1990; Schofield et al., 1987; Unwin, 1993). Current data reflect an extraordinary diversity of $GABA_A$ receptors (referred to as subtypes) formed by combinations of four-five subunit polypeptides, although the exact subunit composition is not yet known for any native

receptor. For example, at least five $GABA_A$-receptor subtypes have been detected in neurons by *in situ* immunofluorescent staining with subunit-specific antibodies (Fristchy et al., 1992). The 16 subunits so far identified are gouped into five classes based on homology of their predicted amino acid sequence : α_{1-6}, β_{1-4}, γ_{1-3}, δ_1, and ρ_{1-2} (Burt and Kamatchi, 1991; Cutting et al., 1992). Members of the same class exhibit 70-80% homology as compared to 30-40% between different classes.

The genes encoding subunits of the $GABA_A$ receptor are differentially expressed depending on the developmental age and tissue type (Fristchy et al., 1992; Laurie, Wisden and Seeburg, 1992; Ymer et al., 1989; Maclennan et al., 1991; Primus et al., 1992). In some brain tissues of the rat, there is a developmental switch from embryonic to adult combinations of subunits, but in others the same subunits are expressed throughout life (Laurie et al., 1992). Moreover, although they are primarily distributed in vertebrate brain, certain subunits are also expressed in non-neuronal tissues (Laurie at al., 1992; Ymer et al., 1989). These observations have raised the possibilities that various modes of GABAergic inhibition are brought about by changes in receptor composition during development and/or in different tissues. This could be a means of fine-tuning neuronal activity (Fristchy et al., 1992; Laurie et al., 1992). Moreover, in non-neuronal tissues, certain $GABA_A$-receptor subtypes might be mediating functions other than neuronal inhibition (Culiat et al., 1993).

Mapping studies have shown that genes encoding $GABA_A$-receptor subunits occur in gene clusters distributed into different human

chromosomes : α5-β3 in chr 15 (Wagstaff et al. 1991; Knoll et al. 1993) , γ1-α2-β1 on chr 4 (Schantz-Wilcox et al. 1992), and γ2-α1 on chr 5 (Schantz-Wilcox et al. 1992). Comparative mapping in mice has localized two of these clusters: α5-β3 on chromosome 7 (Wagstaff et al. 1991; Culiat et al. 1993) and γ1-α2-β1 on chromosome 11 (Buckwalter et al. 1992). We recently mapped the genes encoding the β3 and α5 subunits (*Gabrb3* and *Gabra5*, respectively) on a panel of deletions of the mouse pink-eyed-dilution (*p*) locus and showed a complete concordance between genomic or transcriptional alterations at the *Gabrb3* gene and a usually neonatal-lethal defect in facial development (isolated cleft palate) (Culiat et al., 1993). Recently, the γ3 subunit (*Gabrg3*) was also mapped to a *p*-locus deletion (Nakatsu et al., 1993). Identifying such clusters not only lead to the elucidation of the molecular phylogeny of these $GABA_A$ receptor subunit genes, but in conjunction with expression studies that allow the discernment of patterns of gene regulation, the functional significance of this gene family's organization might be determined. With such a high degree of conserved linkage, mouse models can be readily used for investigating the genomic structure and function of members of this complex gene family.

We hereby report the mapping of *Gabrg3* to a site between *p* (*D15S12h*) and *Gabra5* by analysis of the Oak Ridge series of *p* deletions. Importantly, we describe a phenotypically normal balanced-lethal line of mice carrying overlapping, complementing *p* deletions that fails to express the γ3 transcript, as well as another phenotypically normal line that expresses neither the γ3 nor α5 transcripts. Thus, the absence of γ3

subunit of the $GABA_A$ receptor alone or both $\gamma 3$ and $\alpha 5$ subunits does not result in any overt neurological phenotype in mice.

Materials and Methods

Mice

The origin, maintainance, and analysis of mouse *p*-locus deletions, as well as the breeding strategy to generate *p*-deletion compound heterozygotes, have already been described (Culiat et al. 1993; Nicholls et al., 1993)

DNA and RNA Analyses

Procedures for isolating and analyzing total RNA and DNA by Northern and Southern analysis, respectively, are discussed elsewhere (Nicholls, et. al. 1993), except that washing of blots was done at a stringency of 0.2X standard saline/citrate/0.2% SDS at 68°C for 30 min. PolyA$^+$ RNA fractions were isolated by affinity column chromatography with Mini-Ribosep oligo(dT) columns (Collaborative Biomedical Products, New Bedford, MA).

The derivation of probes for the rat genes *Gabra5* (300/301), and *Gabrb3* (218/219) are described in detail elsewhere (Culiat et al., 1993; Nicholls et al., 1993). The dilute (*d*) gene probe, called C23, corresponds to

nucleotides 1549-3928 of the dilute cDNA sequence and detects three high molecular weight transcripts (12, 8, and 7 kb) in adult mouse brain RNA (Mercer et al., 1991). Hybridization of northern blots with C23 is a control for loading as well as for degradation of high molecular RNAs (such as the 9.5-kb *Gabrg3* transcript). The probe for *Gabrg3* (designated 304/305) was derived by PCR amplification from mouse brain first-strand cDNA templates, synthesized using Amersham cDNA synthesis kit. The primers, 5'TCAAGCTGTCGAAAGCCAAC and 5'GTCCAGCTCAGA GACATCAA, amplify a 306-bp fragment extending from nucleotides 1046-1352 in the published mouse cDNA sequence (Wilson-Shaw et al., 1991). This sequence corresponds to a region predicted to code for the putative intracytoplasmic domain of the subunit polypeptide. This region display little homology with other subunits of the same class, thereby, minimizing cross-hybridization. PCR conditions used were 95°C, 4 min; 36 cycles of 94°C, 30 secs/ 55°C, 1 min/ 65°C, 3 min; 72°C, 4 min. All reagents were from Perkin-Elmer. All PCR products were purified by affinity chromatography using Stratagene Primerase Quik Kit.

Results

Deletion Mapping of *Gabrg3*

We previously reported that the genes encoding the α5 and β3 subunits of the $GABA_A$ receptor map distal to *p* in chromosome 7 in the

order *p-Gabra5-Gabrb3* map (Culiat et al. 1993; Nicholls et al. 1993). The positioning of *Gabrg3* in this chromosomal segment was accomplished by hybridization of the probe 304/305 to the same Southern blots containing various DNAs from 20 *p* deletion mutants that were used to map the other subunit genes.

The $p^{4THO-II}$ mutant breaks inside *p* and extends distally past *Gabrb3* (Nicholls et al., 1993). The absence of two *Pvu*II fragments, 23.0 and 6.6 kb in size, recognized by 304/305 indicated that $p^{4THO-II}$ deletes the *Gabrg3* gene (**Fig. 1a**). Each of 10 other mutations that delete *Gabrb3* and *Gabra5* (p^{8R250M}, p^{47DTD}, p^{2HATh}, p^{45DTD}, p^{116G}, p^{132G}, p^{30PUb}, $p^{23DFiOD}$, p^{55PB}, and $p^{7FR60Lb}$) also delete *Gabrg3*. Out of 6 *p* mutations that do not delete *Gabra5* and *Gabrb3* , four (p^{46DFoD}, p^{6H}, p^{8OK}, p^{4R250H}) delete *Gabrg3* while the remaining two (p^{25DVT}, $p^{12R30Lb}$) do not (**Fig. 1a**). **Fig. 1b** summarizes these deletion-mapping data and show that these are consistent with the order *p-Gabrg3-Gabra5-Gabrb3*. Other investigators have previously mapped *Gabrg3*, and *Gabra5* between *p* and *Gabrb3* but no data regarding to their order were presented (Nakatsu et al. 1993).

Mutants Deleted for γ3 Alone or for Both α5–γ3 Subunit Genes are Phenotypically Normal

The deletion map (**Fig 1b**) shows that certain compound heterozygotes with deletions that complement for prenatal-lethal factors

mapping proximal to *p* (Culiat et al.1993; Nicholls et al. 1993; Lyon et al. 1992) will have genotypes that completely delete certain $GABA_A$ subunit genes. For example, the genotype $p^{46DFIOD}/p^{4THO-II}$ is deleted for both copies of *Gabrg3*, whereas $p^{83FBFo}/p^{4THO-II}$ is deleted for both *Gabrg3* and *Gabra5*. Surprisingly, these mice are completely viable and fertile. Other than the pink-eyed phenotype, which results from the absence of both *p* alleles in these genotypes, the mice appear normal. There are no obvious abnormalities in both brain morphology and behaviour. Since *Gabrg3* is expressed in developing intestine (Laurie et al., 1992), mice homozygously deleted for *Gabrg3* were examined for defects in the stomach and intestines. No readily detectable abnormalities were observed (data not shown).

To confirm that there were indeed no expression of the genes in mice with the interesting genotypes, $p^{46DFIOD}/p^{4THO-II}$ and $p^{83FBFo}/p^{4THO-II}$, probes for *Gabra5* and *Gabrg3* were hybridized to blots containing adult brain polyA$^+$ RNA. As expected, the 9.5-kb wild-type γ3 transcript is absent from both lines while the wild-type 2.5-kb α5 message is absent in $p^{83FBFo}/p^{4THO-II}$ and present in $p^{46DFIOD}/p^{4THO-II}$ (**Fig. 2a and b**).

Gabra5 and *Gabrg3* are not Imprinted in Mice

The absence of an overt aberrant phenotype in mice heterozygous for the $p^{4THO-II}$ deletion (i. e. $+/p^{4THO-II}$) inherited from either parent

suggests that neither *Gabrg3* nor *Gabra5* is functionally imprinted, similar to the situation found for *Gabrb3* (Nicholls et al., 1993; Cattanach et al., 1992). Analysis of poly A^+ RNA from adult mouse brain (**Fig. 2c**) shows that the corresponding transcript is expressed equally well from the maternal chromosome (where the $p^{4THO\text{-}II}$ deletion is inherited from the sire) or from the paternal chromosome (where the deletion is inherited from the dam).

Discussion

It is interesting that all $GABA_A$ subunit clusters so far encountered are composed of subunits from different classes instead of members of the same class arranged in tandem. Patterns of gene regulation of subunit genes may give light to the functional significance of this type of organization. So far, no two subunit genes display identical temporal and spatial patterns, although in brain, the α2, α3 and α5 genes are often expressed during the same time and in the same tissues as β3 (Fristchy et al., 1992). Despite an extensive study of the expression in rat brain of 13 out of the 15 known subunit genes, there is no evidence for the functional importance (eg., coordinate regulation, sequential switching during development) of the clustered organization of subunit genes.

Analysis of mice that carry overlapping, complementing deletions of the *p* locus in chromosome 7 demonstrated that both γ3 and α5 subunits of the $GABA_A$ receptor are dispensable for survival and that their absence

does not cause any severe neurological defect or other aberrant phenotypes. The mice missing γ3 function ($p^{46DFIOD}/p^{4THO-II}$) or both α5 and γ3 functions ($p^{83FBFo}/p^{4THO-II}$) are currently being evaluated for subtle behavioural or pharmacological abnormalities. It is known that a point mutation in the gene encoding the the α6-subunit polypeptide is associated with alcohol intolerance in rats, in which animals are highly susceptible to impairment of postural reflexes by alcohol and benzodiazepines (Korpi et al., 1993). Recent data indicate that in the Harwell mutation, p^{cp} (which is deleted for *Gabrg3-Gabra5-Gabrb3*), dramatic alterations of ligand-binding properties of the receptor in brain occur (Nakatsu et al., 1993). A comparative study of receptor function in $p^{83FBFo}/p^{4THO-II}$ and $p^{46DFIOD}/p^{4THO-II}$ can provide excellent systems to dissect out the function of these particular α, β and γ subunits of the $GABA_A$ receptor.

Disruptions in *Gabrb3* expression, however, are completely concordant with neonatally lethal cleft palate (Culiat et al., 1993). Based on these findings and on earlier teratological studies (Wee and Zimmerman 1983); Zimmerman and Wee, 1984), we hypothesized that deficiency of the β3 subunit during facial development may be responsible for the clefting defect (Culiat et al., 1993). It is possible that failure to express a normal β3 transcript may also contribute to the variable neurological phenotype (i. e. tremor and jerky gait) exhibited by those rare $p^{4THO-II}$ (Culiat et al., 1993) or p^{cp} (Nakatsu et al., 1993) homozygotes that escape neonatally lethal cleft palate. On the other hand, the normal phenotypes exhibited by mice deleted for γ3 or both α5 and γ3 subunit genes are in

conflict with a recent report implicating these genes in the neurological defects of these rare escapers (Nakatsu et al., 1993).

Intercrossing of $p^{83FBFo}/p^{4THO-II}$ compound heterozygotes creates a balanced-lethal line in which both homozygous classes die prenatally, owing to deletion of a lethal factor mapping proximal to *p* (L. B. R. and C. S. M., unpublished data), and greater than or equal to 95% of $p^{4THO-II}/p^{4THO-II}$ neonates die at birth from cleft palate (Culiat et al. 1993). Thus, such a cross leaves only the next generation of $p^{83FBFo}/p^{4THO-II}$ compound heterozygotes that are deleted for the γ3 and α5genes. The availability of completely viable and fertile mutant mouse strains that are deleted for both the γ3 and α5 subunits of the $GABA_A$ receptor (or for the γ3 subunit alone, as with $p^{46DFiOD}/p^{4THO-II}$ mice) will make unnecessary the creation of null mutations by targetted mutagenesis of embryonic stem cells. Due to the role of GABA as an inhibitory neurotransmitter, mutations in $GABA_A$ receptor subunit genes have been postulated to cause neurogenetic diseases as diverse as alcoholism, manic depression, and Angelman syndrome (Wilcox et al., 1992; Wagstaff et al., 1991). Genetic knock-outs of specific subunit genes will confirm these hypotheses, but so far there has been no report of targeted mutagenesis of any of the 15 $GABA_A$ subunit genes.

These mouse lines deleted for *Gabrg3* ($p^{46DFiOD}/p^{4THO-II}$) or both *Gabrg3* and *Gabra5* ($p^{83FBFo}/p^{4THO-II}$) are useful models for investigating the role(s) of receptor subtypes in the mammalian nervous system, as well as in other tissues of the developing fetus. These lines are particularly useful in the analysis of the organismal functions of the γ3 and

α5 subunits by providing large numbers of animals for a wide range of behavioral and pharmacological studies. In contrast, only one in 80 to one in 100 mice obtained from intercross of heterozygotes carrying the p^{cp} deletion (Nakatsu et al., 1993; Lyon et al., 1992; Phillips, 1973; Brilliant, 1992) or the $p^{4THO\text{-}II}$ (Culiat et al., 1993) respectively will be homozygous for the deletion at *Gabrg3* and *Gabra5* and be viable. Furthermore. these rare survivors are not very fit and die by weaning (Culiat et al. 1993; Lyon et al. 1992; Phillips, 1973; Brilliant, 1992).

The localization of neurotransmitter receptor subunit genes near *p* is intriguing because the corresponding region in humans is associated with Angelman syndrome (AS), a severe disorder characterized by seizures, uncontrolled laughter, absence of speech, stiff jerky movements and severe mental retardation (Nicholls, 1993; Kuwano et al. 1993). AS is produced by maternally inherited mutations in 15q11-q13 or by paternal uniparental disomy of this chromosome (Nicholls, 1993; Kuwano et al., 1993, Greger et al., 1993). Because of a potential relationship between defects in $GABA_A$-receptor genes and AS in humans (Wagstaff et al., 1993; Saitoh et al., 1992), we tested whether *Gabra5* and *Gabrg3* are functionally imprinted in mouse, and now present data that they are not, just like *Gabrb3* (Nicholls et al. 1993; Cattanach et al., 1992). However, it is important to note that imprinting is not always conserved between species as demonstrated by *Igf2r*, which is imprinted in mice but not in humans (Kalscheuer et al. 1993). Furthermore, imprinting of expression is not necessarily uniform from tissue to tissue (DeChiara et al. 1991). However, even taking these points into consideration, the absence of

neurological phenotypes in mice deleted for γ3 or both α5 and γ3 make them inappropriate models for the AS syndrome, as was suggested by an earlier study (Nakatsu et al. 1993).

Genetic- and physical-mapping studies have suggested that the order of loci in human 15q11-q13 is centromere-*ZNF127(D15S9)-(ANCR, GABRB3)- GABRA5-D15S12 (P)*-telomere (Knoll et al. 1993; Kuwano et al. 1992; Greger et al. 1993). The existence of an AS patient (Reis et al. 1993) who carries an unbalanced translocation and is hemizygous for loci proximal to *GABRB3* but is heterozygous for a polymorphism in the 3' (proximal) end of the *GABRB3* gene suggests that *GABRB*3 itself may not be involved in AS, although it is still not certain whether the β3 subunit is indeed expressed in this patient. The concordance between genomic or transcriptional alterations at the *Gabrb3* locus and cleft palate in mice (Culiat et al. 1993) provides additional, but not yet conclusive, evidence that this gene is involved in facial development but not in AS. Interestingly, Cattanach et al. (1992) have suggested that there may not be a paternal-duplication imprinting phenotype in mouse chromosome 7 that is reflective of the human AS phenotype. Comparison of the gene order in mouse of centromere-*D15S12(p)-Gabrg3-Gabra5-Gabrb3-Znf127 (D15S9h-1)*-telomere with that in humans suggests that if mouse models for AS can be identified, they will probably arise from mutations in genes (whether imprinted or not) that map distal to *Gabrb3* (**Fig. 1b**).

SECTION III.

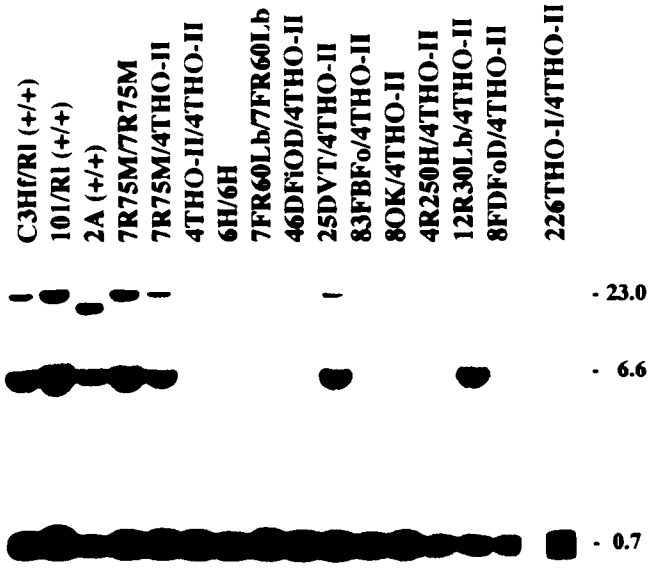
FIGURES

FIGURE 1. Mapping *Gabrg3* to the *p-Gabra5* Interval.

(**A**) A Southern blot of 10 μg of *Pvu*II-digested DNA from animals of the indicated genotypes was hybridized to probe 304/305, which detects 23- and 6.6-kb genomic fragments at the *Gabrg3* locus. The genotypes are abbreviated; for example, "4THO-II/4THO-II" refers to $p^{4THO\text{-}II}/p^{4THO\text{-}II}$. Note that *Gabrg3* is not deleted in p^{25DVT}, which deletes the entire *p* gene (Rinchik et al., 1993). *Gabrg3* is deleted in p^{83FBFo}, which is also deleted for *Gabra5*, and in four mutations, $p^{46DFiOD}$, p^{6H}, p^{8OK}, and p^{4R250H} that do not delete *Gabra5*. Hybridization of the same blot to the control probe 34-1-111, from the *D15S9h-1* locus detects 4.1-kb and 0.7-kb *Pvu*II fragments but only the latter is shown. A polymorphic fragment of ~17.0 kb is also detected by the *Gabrg3* probe, but is irrelevant to this analysis.

(**B**) A map of chromosome 7 integrating the position of *Gabrg3* relative to other previously mapped markers around the *p* locus. The open circle at the left represents the centromere. The abbreviated name of each deletion is written above the line that represents its extent. Endpoints of independently derived deletions that can not yet be differentiated by available molecular probes are drawn as terminating at the same point. The p^{cp} deletion begins at the *p* gene and extends distally to end at *Gabrb3* (Nakatsu et al., 1993).

A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101/Rl (+/+)
2A (+/+)
7R75M/7R75M
7R75M/4THO-II
4THO-II/4THO-II
6H/6H
7FR60Lb/7FR60Lb
46DFiOD/4THO-II
25DVT/4THO-II
83FBFo/4THO-II
8OK/4THO-II
4R250H/4THO-II
12R30Lb/4THO-II
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- 23.0
- 6.6
- 0.7

B

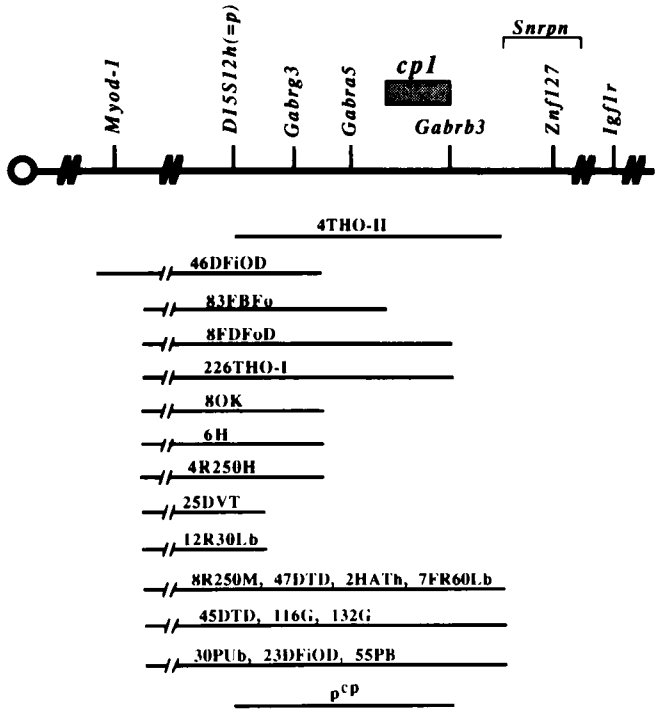
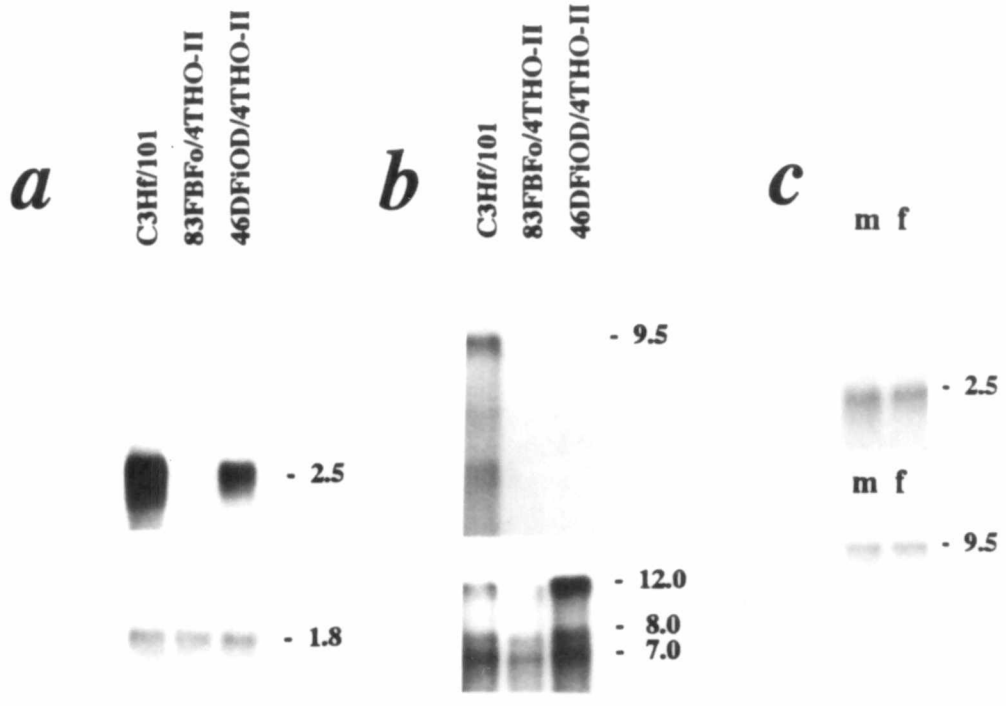

Myod-1
D15S12h(=p)
Gabrg3
Gabra5
cp1
Gabrb3
Snrpn
Znf127
Igf1r
4THO-II
46DFiOD
83FBFo
8FDFoD
226THO-I
8OK
6H
4R250H
25DVT
12R30Lb
8R250M, 47DTD, 2HATh, 7FR60Lb
45DTD, 116G, 132G
30PUb, 23DFiOD, 55PB
pcp

FIGURE 2. Expression Analysis of the *Gabrg3* and *Gabra5* Transcripts.

(Blots ***a*** & ***b***) A blot of polyA$^+$ RNA from adult brains of (101/Rl X C3Hf/Rl)F_1, (C3Hf/101); $p^{83FBFo}/p^{4THO-II}$ (83FBFo/4THO-II); and $p^{46DFiOD}/p^{4THO-II}$ (46DFiOD/4THO-II) hybridized with a probe for *Gabra5* (***a***; upper blot) or for *Gabrg3* (***b***; upper blot). The *Gabrg3* probe (304/305) detects a wild-type 9.5-kb transcript that is completely missing from the two mutations. Hybridization of the same blot to the *Gabra5* probe (300/301) shows a 2.5-kb wild-type *Gabra5* transcript, which is absent in the p^{83FBFo} deletion but is present in $p^{46DFiOD}$. As a control for the amount of RNA loaded in each lane, the blot was hybridized to either chicken tubulin (1.8-kb transcript in ***a***) or the chromosome 9 dilute gene (12, 8, and 7 kb in ***b***). The sizes of transcripts were determined by comparison to RNA ladder (Life Technologies). The decrease in signal of the 2.5-kb *Gabra5* transcript in $p^{46DFiOD}/p^{4THO-II}$ is due to hemizygous expression of the gene from the $p^{46DFiOD}$ chromosome (*Gabra5* is completely deleted in $p^{4THO-II}$)

(Blot ***c***) Probes for *Gabra5* (300/301) and *Gabrg3* (304/305) were also hybridized to an RNA blot containing poly A$^+$ RNA from adult brains of heterozygotes inheriting the $p^{4THO-II}$ deletion from either the male (m) or female (f) parent. Both genes are expressed from either parent.


a
C3Hf/101
83FBFo/4THO-II
46DFiOD/4THO-II
- 2.5
- 1.8
b
C3Hf/101
83FBFo/4THO-II
46DFiOD/4THO-II
- 9.5
- 12.0
- 8.0
- 7.0
c
m f
- 2.5
m f
- 9.5

IV. PHYSICAL MAPPING OF THE *cp1* INTERVAL

Introduction

A cluster of genes encoding the γ3, α5, and β3 subunits of the $GABA_A$ receptor (*Gabrg3*, *Gabra5,* and *Gabrb3*, respectively) was recently mapped distal to the pink-eyed dilution locus (Nakatsu et al., 1993; Culiat et al., 1994). There are several compelling reasons for determining the physical map surrounding these loci in mouse chromosome 7: (a) *Gabrb3* is a candidate gene for *cp1*, a gene(s) controlling secondary palate development (Culiat et al., 1993); (b) disruption of a gene in the region between *Gabra5* and *Gabrb3* may lead to growth retardation (Culiat et al., 1994); (c) since three $GABA_A$ receptor genes have been mapped to this region (Culiat et al., 1994; Nakatsu et al., 1993) it is possible that another subunit gene may be located nearby. This gene could provide another candidate for *cp1* or for neurological phenotypes associated with this region (Culiat et al., 1994; Nakatsu et al., 1993); and (d) comparison of the physical map of these loci with the established homologous region in human chromosome 15 might give insights into the characteristic genomic organization of $GABA_A$ receptor subunit genes.

Genetic complementation, gene expression, and teratological analyses strongly suggest that *Gabrb3* is *cp1*, a gene that controls mouse secondary palate development (Culiat et al., 1993). The genetic interval containing *cp1* was initially defined to be distal to the distal breakpoint of

the *p* deletion, p^{83FBFo}, extending to the *Gabrb3* gene. Since the physical size of this interval is unknown, it could conceivably contain other genes that are potential candidates for *cp1*. For instance, both the teratological and genetic data would still be consistent if another $GABA_A$ receptor subunit gene, within the defined interval, is responsible for the *cp1* phenotype. The candidacy of *Gabrb3* will therefore be reinforced if the genetically defined interval is physically small. On the other hand, if the interval is large it might become imperative to search the area aggressively for other candidate genes.

Mice that are deleted for the entire *Gabrg3-Gabra5-Gabrb3* cluster usually die from cleft palate, but rare survivors have been observed at a frequency of 1/80 for the p^{cp} mutation and 1/100 for the $p^{4THO-II}$ deletion (Phillips, 1975; Culiat et al., 1993 and 1994; Nakatsu et al., 1993). These survivors have been described as runted and nervous. Genetic complementation analysis has already ruled out any gene from *p-Gabrg3-Gabra5* interval as being responsible for this phenotype. Transgenic $p^{4THO-II}/p^{4THO-II}$ mice rescued from lethal cleft palate (discussed in **section V**) are not nervous, but are still small in size. It is possible, then, that this growth retardation may be accounted for by the disruption of a gene between *Gabra5* and *Gabrb3*. Although mice deleted for both copies of *Gabrg3* or *Gabra5* and *Gabrg3* appear phenotypically normal, they are currently being examined by collaborators for more subtle behavioral aberrations such as learning defects or sensitivity to certain pharmacologically relevant chemicals (eg., those that bind the $GABA_A$ receptor). If any such subtle abnormality exists, it can be attributed to the

deletion of any/or a combination of the subunit genes already found in this cluster or another still unidentified gene.

Mapping of various $GABA_A$ receptor subunit genes revealed that they occur in highly conserved clusters that are scattered over both the mouse and human genomes. The following clusters have been localized in the human genome: (1) *GABRA5* and *GABRB3* map to chr 15 (Wagstaff et al., 1991; Knoll et al., 1993); (2) *GABRG1*, *GABRA2* and *GABRB1* are located on chr 4 (Schantz-Wilcox et al., 1992); *GABRG2*, *GABRA1* and *GABRA6* and *GABRB2* into chr 5 (Schantz-Wilcox et al., 1992; Hicks et al., 1994: Russek and Farb, 1994); and *GABRR1* and *GABRR2* into chr 6 (Cutting et al., 1992). In mouse, *Gabrg3*, *Gabra5* and *Gabrb3* map to chr 7 (Culiat et al., 1993; 1994), and *Gabrg1*, *Gabra2* and *Gabrb1* map to chr 11 (Buckwalter et al., 1992). The biological significance of this clustered organization remains to be elucidated. Genes in the *Gabrg3-Gabra5-Gabrb3* cluster are all expressed in high levels in mouse brain in embryonic development and in the early post-natal period (Laurie et al., 1992). It is therefore possible that a clustered organization might facilitate coordinate regulation. This has been suggested for the *GABRA1*, *GABRB2* and *GABRG2* cluster because these subunit polypedtides comprise the most common combination of expressed subunit genes (Wilcox et al., 1992; Russek and Farb, 1994). Since most clusters identified so far have at least α-β-γ subunit genes clustered, it has been proposed that duplication and translocation of an ancestral α-β-γ cluster to other chromosomes gave rise to the various clusters that are now being identified (Russek and Farb, 1994).

For the reasons discussed above, a physical map of the *cpl* interval was established based on pulsed-field gel electrophoresis (PFGE) of wild-type genomic DNA and restriction mapping of overlapping mouse genomic fragments covering the *cpl* interval recovered as yeast articifical chromosomes (YAC clones). In addition to resolving the size of the region where *cpl* has been localized, a detailed physical map will provide a basis for selecting which techniques are likely to be the most effective in finding other genes in the area (reviewed by Stubbs, 1992).

PFGE analysis involves the generation of large genomic fragments by digesting DNA with restriction enzymes that cut rarely in the genome. The low frequency of these "rare-cutting enzyme" sites is due to two factors: (1) the enzymes are sensitive to the methylation status of the recognition site; and (2) the sites contain the CpG dinucleotide, which is underrepresented in the mammalian genome (Barlow and Lehrach, 1987). PFGE analysis permits direct measurement of the chromosomal distance between closely-linked markers by demonstrating their presence or absence within the same or overlapping large restriction fragments. Different combinations of enzyme digests (i.e., double digests) can confirm and refine the position of the markers. Clusters of these rarecutting enzyme sites (referred to as CpG islands) are frequently found near the 5' region of mammalian genes (Bird, 1987; Stubbs, 1992). The localization of these islands in a stretch of genomic DNA thus provides landmarks for the position of genes in a region of interest.

Materials and Methods

Southern Analysis and Probes

The following PCR-derived probes were used in the analyses presented in this section: 218/219 (3' β3), 280/281 (5' β3), 300/301 (3' α5), 384/385 (5' α5) and 304/305 (3' γ3). Derivation of 300/301, 218/219 and 304/305 have been described elsewhere (Nicholls et al., 1993; Sections II and III). **Tables 1 and 2, General Appendix** summarize the characteristics of all these probes.

PCR probes were synthesized on either rat- or mouse-brain first-strand cDNA. These templates were made by reverse transcription of 10 μg of total RNA using random hexanucleotides from the Amersham cDNA synthesis kit and catalyzed by murine moloney leukemia virus reverse transcriptase (total volume of 25 μl). Each primary PCR reaction was in a 100 μl volume consisting of 1.0 μl of reverse transcription reaction, 200 nanograms each of primers, 5 μl of 25 mM $MgCl_2$, 10 μl 10X PCR buffer, 10 μl 1 mM dNTPs, with the rest of the volume made up with distilled water. PCR conditions used were 95°C, 4 min; 36 cycles of [94 °C, 30 secs/ 55 °C, 1 min/ 65 °C, 3 min]; 72 °C, 4 min. All PCR amplifications were conducted in a Perkin Elmer Cetus GeneAmp™ PCR System 9600 machine. To synthesize enough product for use as probe, re-amplifications were done on 1-μl aliquots of the primary PCR reaction, using the same program and reaction mix but with half the Mg^{++}

concentration in order to increase specificity. Re-amplication reactions were pooled, and the desired PCR fragment was purified either by affinity chromatography using the Stratagene Primerase Quik Kit or by electrophoresis and subsequent extraction from agarose slices. The latter was used only when the PCR reaction had products in addition to the desired fragment.

The probe 280/281 is a 490-bp fragment extending from nucleotides 81- 570 of the *Gabrb3* cDNA (Ymer et al., 1989) and detects fragments at the 5' portion of the *Gabrb3* gene. The predicted amino acid sequence of the 280/281 DNA sequence extends from the signal peptide to the first half of the β structural loop (Ymer et al., 1989). The primers used for this probe were 5'-TTGCGGGAGGAAGGCTTTTC-3' and 5'-CTTCTGAGGTCCATCATGCA-3'. PCR amplification was done on mouse-brain cDNA.

The probe for the 5' region of *Gabra5* (385/386) is a 128-bp fragment stretching from nucleotides 207-335 and was amplified from mouse cDNA using the primers: 5'-GCTTGCTGGCTTTGAACGTG-3' and 5'-GCATTCCATTGTCCATTCCCAG-3'. This fragment extends 113 bp upstream until 15 bp downstream of the start codon. The primers were designed based on the sequence from the gene bank accession *X51992*; locus *RNGABAA5*. DNA analysis that further defined the breakpoint of p^{83FBFo} was done by hybridization of 385/386 to the deletion panel used for mapping *Gabra5* and *Gabrb3* as previously reported (Culiat et al., 1993).

Analysis of the *cp1* Interval by Pulsed-field Gel Electrophoresis

Standard protocols and their underlying principles for analyzing genomic DNA by pulsed-field gel electrophoresis are described elsewhere (Anand, 1986; Lindsay and Bird, 1987; Barlow and Lehrach, 1987). The modifications of these procedures that were employed in this study are described by Stubbs et al. (1994, in press).

High molecular weight mouse DNA was prepared in agarose blocks for pulsed-field gel electrophoresis (PFGE). Spleen cells from wild-type C3Hf/Rl mice were isolated and resuspended in phosphate-buffered saline, and then embedded in agarose by addition of an equal volume of 1.5% low melting agarose (INCERT) so each 100 µl block would have ~10^6 cells. Blocks were incubated in 0.5M EDTA-1% sarkosyl with 1 mg/ml proteinase K for two days at 50 °C. Before digestion with restriction enzymes, the blocks were incubated in 40 µg/ml phenylmethylsulfonyl fluoride (PMSF) at 50 °C, followed by two washes in Tris-EDTA buffer.

DNA was digested into large fragments using the following enzymes from Boehringer Mannheim and New England Biolabs: *Not*I *(N), Nru*I *(R), Eag*I, *(E), Mlu*I *(M), Bss*HII *(B), Ksp*I *(K), Sal*I *(S)* and *Xho*I *(X)*. DNA digests with these rare-cutting enzymes were conducted for 12-16 hrs using conditions specified by the manufacturer. The digested DNA in agar blocks were loaded into 1% Seakem LE agarose gels, cast and ran in 0.5 X TBE buffer (0.045 M Tris, 0.045 M Boric Acid, 0.001 M EDTA). All of the electrophoreses were done in a Biorad Chef-mapper apparatus using an autoalgorithm program that sets the other parameters based on the

minimum and maximum fragment sizes to be resolved, at 13 °C. After staining the gel in 1μg/ml ethidium bromide, the DNA was nicked by UV-irradiation at 30,000 μjoules/m^2 in a Stratalinker 2400 unit (Stratagene, La Jolla, CA), incubated in alkali (0.5M NaOH, 1.5 M NaCl) for ~2 hrs and then blotted in the same alkali solution onto Duralon nylon filters for two days. The filters were neutralized by briefly rinsing in 50 mM sodium phosphate buffer (pH 7.0), baked in vacuum at 80 °C for 1-2 hrs, and then immediately crosslinked at 25, 000 μjoules/m^2. Probes for known genetic markers around the *cp1* interval were hybridized to these southern blots. Sizes of restriction fragments were compared to the following commercial molecular weight markers: yeast chromosomes; middle and low range markers (λ concatemers and λ *Hind*III, respectively).

YACs Covering the *Gabra5-Gabrb3* Region

The analysis of large segments of the mammalian genome is possible by cloning genomic fragments, measuring hundreds of kilobases in length into vectors that permit the propagation of these clones as artificial yeast chromosomes (called YACs) in yeast cells (reviewed by Hieter, 1990). All the YACS that were analyzed in this section were obtained from the Imperial Cancer Research Fund (ICRF) Mouse YAC library (Larin, Monaco and Lehrach, 1991). The ICRF YAC library was constructed from *Eco*RI partially digested C3Hf/Rl (+/+) DNA cloned into the *pYAC4* vector (ura^+trp^+). YAC clones are used to transform AB1380 (ura^-trp^-)

yeast cells and then grown in selective media without uracil and tryptophan. Protocols for construction of YAC libraries, preparing YAC DNA for analysis, and discussions on the applications of YACS to the analyses of large genomic fragments are described elsewhere (Barlow and Lehrach, 1987; Hieter et al., 1990; Schlesinger, 1990; Wicking, 1991; Stubbs, 1992).

YACs spanning the *cpl* interval were identified by screening replica filters of the ICRF Mouse YAC library (Larin et al., 1991) with the probes 218/219 (3' *Gabrb3*) and 300/301 (3' *Gabra5*). Both are sequences corresponding, respectively, to the putative intracytoplasmic domain of *Gabrb3* (Ymer et al., 1989) and *Gabra5* (Krestchatisky et al., 1989). This domain is the least conserved sequence of $GABA_A$ receptor subunit genes, thereby reducing the chances that a homologous subunit gene will be detected. Probe 218/219 hybridized to two YACs, YGO6110 and YB0392, while 300/301 picked up YGO713 and YG0813. Wild-type C3Hf/Rl and YAC DNA (embedded in agar blocks) were digested with *Eco*RI and *Bam*HI, electrophoresed on agarose gels and blotted onto Duralon nylon filters. These were hybridized to the same probes used to initially select the YAC clones in order to assure that the probes detected the same size genomic fragment on both the control and YAC DNA. Probes for the 5' region of *Gabrb3* (280/281) and *Gabra5* (385/386) were also hybridized to these blots.

Endcloning of YACS

Except for the left arm endclone of YGO713, all endclone probes used in this study (summarized in **Table 1, General appendix**) were derived by an inverse polymerase chain reaction protocol modified from Kere et al. (1992) and Mueller and Mold (1989). To obtain the left end, YAC DNA in agarose blocks was digested with *Taq*I, *Rsa*I, or *Eco*RV. For the right end *Pst*I, *Rsa*I or *Eco*RV digests were used. The DNA fragments were circularized by ligation, then amplified by PCR using primer sequences from either the right or left arm of *pYAC4*. PCR conditions used were 95°C, 4 min; 30 cycles of (94 °C, 30 sec/ 60°C, 30 secs/ 72°C, 1min and 30 secs); 72 °C, 4 min. The endclone was then reamplified by PCR then purified by column chromatography using the Stratagene Primerase Quik Kit.

A plasmid rescue protocol (Schlesinger, 1990) was applied to obtain the left end of YG0713. YAC DNA was digested with *Bam*HI then religated. If *Bam*HI cuts within the cloned mouse genomic DNA and within the left arm of *pYAC4*, which contains the gene for ampicillin resistance (Amp^R), a recircularized fragment that contains both vector and mouse sequences will be Amp^R. The recircularized fragment was then used to transform ampicillin-sensitive *E. coli* cells by electroporation. Amp^R colonies are then selected and plasmid DNA was isolated and purified by standard techiniques. Plasmid DNA was digested and run in agarose gel. The fragment was cut out from the agarose gel and purified by treating the agar slice with β-agarase (New England Biolabs) and

extracting the DNA by phenol-chloroform extraction protocols.

To minimize hybridization of the YAC endclone probe to repetitive mouse and/or yeast DNA sequences, these probes were pre-associated with total mouse DNA and *Eco*RI-digested *pYAC4* DNA using the conditions described by Stubbs et al. (1990).

Restriction Mapping of YGO6110 and YGO713

Partial *BssH*II, *Mlu*I, *Eag*I, *Not*I and *Nru*I digests of YACs were generated by digesting YAC DNA with varying enzyme concentrations and incubation times. Conditions used for *BssH*II were A) 0.1 Units (U), 30 mins; B) 0.3 U, 30 min; C) 1.0 U 30 mins and D) 4.0 U, 2 hrs. *Mlu*I and *Eag*I were digested using the conditions : A) 0.2 U, 30 mins; B) 0.4 U, 30 mins; C) 1.0 U, 30 mins; D) 10 U, 2 hrs. Finally, *Not*I and *Nru*I conditions were: A) 2.0 U, 2 hrs B) 5.0 U, 2 hrs C)10 U, 2hrs D) 10 U, 6 h-overnight. Electrophoresis and blotting procedures were the same as those described for the PFGE analysis of wild-type genomic DNA described earlier in this section.

Blots containing partial digests of YAC DNA were hybridized to probes specific for either arm of *pYAC4*. YGO6110 blots were probed with the *Sph*I-*Nru*I fragment from the right arm of *pYAC4* and the *Acc*I-*Pvu*II fragment from the left arm of *pYAC4*. To reduce the background bands from hybridization of *pYAC4* arm probes to other yeast sequences, YG0713 blots were hybridized to the *Pvu*II-*Bam*HI fragments of pBR322

that corresponds to either left or right arm of *pYAC4*. Hybridization with *Gabrb3* probes 218/219 and 280/281, and *Gabra5* probes 300/301 and 385/386 were also performed to incorporate these markers into the physical map.

Subcloning of YGO713 into Cosmids

DNA was prepared from an overnight 500-ml liquid culture of *YGO713* incubated at 30 °C. The yeast cells were collected by centrifugation and then resuspended in 50 mM EDTA, 10mM Tris. After a second centrifugation the cells were resuspended in Sorbitol-Citrate-EDTA buffer. Cell walls were digested by treating with yeast lytic enzyme (ICN Biochemicals Inc). Proteinase K was added to a final concentration of 100 µg/ml, with incubation at 55 °C for 2 hrs. The DNA was then isolated by a series of phenol and chloroform extractions. Further purification of the aqueous solution of YGO713 DNA was done by dialysis in two changes of 2 l of Tris-EDTA buffer at room temperature and a final change at 4 °C, overnight. The DNA was precipitated with ethanol and finally resuspended in TE buffer (10mM Tris, 1.0 mM EDTA).

An *Mbo*I partial digest of aqueous YGO713 DNA was made by adding 0.05 Units of enzyme per µg DNA into a 1250 µl reaction containing 50 µg DNA. Two 500 µl aliquots and a final 250 µl aliquot were removed from the reaction at 2, 4, and 6 mins and digestion was terminated by heat inactivation. The extent of digestion of the aliquots

was assessed by running 20 µl in a minigel. The *Mbo*I partials were fractionated in a sucrose gradient and 500 µl fractions were collected. After checking the average size of DNA fragments in each fraction by electrophoresis, the fraction containing ≥ 23 kb fragments was cloned into the *Bam*HI site of the cosmid vector *pCOS2,* which was first linearized with *Pvu*II, de-phosphorylated, then digested with *Bam*HI and de-phosphorylated again. Ligated DNA was packaged using commercial reagents (Stratagene Gigagold) and then used to infect NM554 Mg^{++} bacterial cells. Transformed colonies were selected in medium containing kanamycin. The library was plated at a density of 50,000-60,000 clones per plate. Replicas of the colonies were lifted into Magna Nylon Transfer Membrane (Micron Separations Incorporated), regrown on top of a fresh LB plate, denatured in alkali, neutralized, then rinsed in 50 mM sodium phosphate buffer. Filters were baked at 80 °C, and immediately crosslinked with UV at 25,000 µjoules/m^2. Cosmids containing *Gabrb3* and *Gabra5* were identified by hybridization with 280/281 (5' *Gabrb3*) and 300/301 (3' *Gabra5*), respectively. B1 and B2 mouse repetitive DNA were also used as probes to identify other clones in the library that contain mouse sequences.

Results

Distal Breakpoint of p^{83FBFo} is Within the Gene Encoding the $\alpha 5$ Subunit of the $GABA_A$ Receptor (*Gabra5*)

Hybridization of a probe corresponding to the 5' coding region of *Gabra5* (385/386) to a Southern blot containing DNAs from the various *p* deletions (**Fig. 1A**) indicated that the deletion p^{83FBFo} does not remove this probe sequence. The pattern of hybridization with respect to the other *p* deletions was identical to that of the 3' *Gabra5* probe, 300/301 (Culiat et al., 1993). Since it was previously reported that the p^{83FBFo} mutation is deleted for 300/301, the distal breakpoint of p^{83FBFo} must be within the *Gabra5* transcription unit and that the transcriptional orientation of this gene is in the opposite direction from *Gabrb3* (**Fig. 1B**). These data imply that the *cp1* interval begins immediately distal to the *Gabra5* gene. Earlier genetic and molecular analyses indicated that the *cp1* interval does not extend beyond *Gabrb3* (Culiat et al., 1993); hence, the physical distance between the genes *Gabra5* and *Gabrb3* defines the maximum size of the genetically defined *cp1* interval.

PFGE Map of the *Gabra5-Gabrb3* Region

The *Gabrb3* probe 218/219 detects 1200-kb fragments in digests generated by almost all rare-cutting enzymes used (**Fig. 2**). Double digests

of either *Not*I or *Nru*I with other rarecutters also generate the same size fragment, indicating that sites for these enzymes are located very close to each other on both ends of the long fragments. It is possible, then, that these fragments are generated by cutting at two strong CpG islands, located 1200 kb apart. When a probe (280/281) corresponding to the 5' end of the *Gabrb3* gene was hybridized to a southern blot containing DNA digested with *Bam*HI, as well as double digests of *Bam*HI plus a variety of different rare-cutting enzymes, it detected a *Bam*HI fragment that is cleaved by *Not*I, *Eag*I, *Nru*I, *Ksp*I and *Mlu*I. These results indicate that the 5' end of *Gabrb3* is associated with a strong CpG island containing these enzyme sites. The large fragments detected by 218/219 do not hybridize with probes for other genetic markers that have been mapped around *Gabrb3,* namely, from the *D7Cwr15* locus (0.8 probe); *Gabrg3* (304/305), *Gabra5* (300/301), and *D15S9h-1 or Znf127* (*MuDN34*). Mapping by PFGE of the markers *D7Cwr15, Gabrg3* and *D15S9h-1* were done in collaboration with L. Stubbs (unpublished data).

The 3' *Gabra5* probe (300/301) detects smaller rare-cutting enzyme fragments 300 kb in size (**Fig. 2**). Unpublished data by L. Stubbs show that 300/301 also hybridizes to a ~1800-kb *NruI* fragment and a *Ksp*I partial fragment of the same length that is detected by probes for genetic markers mapped proximal to the *cp1* interval, namely *MN7*, *p*, *0.8* and *Gabrg3*. One of the 300-kb fragments (eg., *Ksp*I) detected by the *Gabra5* probe is one of the series of partial digests that adds up to 1800 kb. These fragments, therefore, physically link *Gabra5* to these other markers.

YACS Spanning the *cp1* Interval

The 3' probe for *Gabrb3* (218/219) detected the YACS YGO6110 and YBO392, measuring ~500 kb and ~530 kb, respectively. Hybridization of the YAC library with a probe (300/301) corresponding to the 3' region of the *Gabra5* gene identified YG0713 and YG0813, both ~430 kb. Based on the similarity in size, identical fragments detected by all probes tested, and identical B1-B2 (Krayev et al., 1982) hybridization patterns on both *Bam*HI and *Eco*RI digests, it was concluded that YGO713 and YGO813 are the same clone. A probe for the 5' coding region of *Gabrb3* (280/281) was hybridized to *Eco*RI-digested DNA from each of the *Gabra5* and *Gabrb3* YACS (**Fig. 3**). Whereas both YGO6110 and YBO392 contain all the three *EcoR*I genomic fragments detected by this probe in mouse genomic DNA (23, 9.5 and 8 kb), YGO713 has only the 23 kb fragment indicating that it contains only a portion of the 5' region of *Gabrb3*. However, YAC YGO713 contains the entire *cp1* interval since it is positive for both α5 and β3 probes.

YBO392 endclones were very repetitive and did not detect fragments missing in any of the deletions. The right end of YGO6110 is deleted in the $p^{4THO\text{-}II}$ and $p^{7FR60Lb}$, deletions but is present in the deletions p^{6H}, $p^{46DFiOD}$, p^{25DVT}, p^{83FBFo}, p^{8OK}, p^{4R250H}, $p^{12R30lb}$, p^{8FDFoD} and $p^{226THO\text{-}I}$ (**Fig. 4**). YGO6110 has the entire *Gabrb3* coding region as indicated by the presence of genomic fragments recognized by of both 5' and 3' *Gabrb3* probes, 280/281 and 218/219 respectively. Since p^{8FDFoD} does not extend to *Gabrb3,* the right end of

YGO6110 must map distal to the *Gabrb3* gene. This positions the left end of YGO6110 proximally, towards *Gabra5*. Surprisingly, the left endclone detects the same fragments in all the *p* deletions and wildtype DNA, suggesting it may be a coligated piece from another part of the genome.

Orientation of YG0713 relative to the *p* deletions was unambiguously established by hybridization of the right end clone to the deletion panel (**Fig. 4**). The right end of this YAC is missing in $p^{4THO-II}$, $p^{7FR60Lb}$, p^{83FBFo}, and p^{8FDFoD} DNA samples but is present in p^{6H}, $p^{46DFiOD}$, p^{25DVT}, p^{8OK}, p^{4R250H}, and $p^{12R30Lb}$. This positions the right end of YGO713 proximally in the interval between *Gabra5* and *p*.

Restriction Map of YGO6110 and YGO713

The restriction maps of YGO6110 and YGO713 are presented in **Figs. 5 and 6.** A strong CpG island is located ~50 kb away from the left end of YGO6110. The enzyme sites clustered in this island are the same as those previously established by PFGE analysis to be in the island near the 5' end of *Gabrb3*. The published rat cDNA sequence of *Gabrb3* shows an *Nru*I site just 50 bp away from the start codon (Ymer et al., 1989). There is only one *Nru*I site in YGO6110 and it is located within the island identified. 5' and 3' probes for *Gabrb3* detect the 400-kb, but not the 100-kb, *Nru*I YAC fragment of YGO6110 (**Fig. 7**). The sites in this island are consistent with those found in the PFGE analysis of wild-type genomic DNA. All these data confirm that the strong island in YGO6110 is

associated with the 5' region of *Gabrb3*.

Hybridization of the 5' probe for *Gabrb3* (**Fig. 8**) and 5' and 3' probes for *Gabra5* (**Fig. 9**) to partial digest blots of YGO713 allowed us to deduce that the CpG island located 180-200 kb from the right end corresponds to the 5' end of *Gabra5*. We propose that the island located ~295 kb from the right end of YGO713 is the *Gabrb3* island, because, (1) the sites defining that island are identical to those shown to correspond to the 5' end of the *Gabrb3* gene in YGO6110 (**Fig. 5**) and also in genomic DNA blots; and (2) hybridization with 280/281 probe (5' β3) to partial digests of YGO713 detects a pattern of *Bss*HII fragments (**Fig. 8**) consistent with the probe sequence being located right next to this island (**Fig. 5**). Consequently, *Gabra5* and *Gabrb3* are ~100 kb apart. The restriction maps of YGO6110 and YGO713 and the position of probes for *Gabra5* and *Gabrb3* are illustrated in **Fig 10**. YAC and PFGE data are integrated in **Fig. 11**.

Cosmids Containing *Gabrb3* and *Gabra5* Sequences

Four cosmids were detected with 280/281 (*5' Gabrb3*), in the subgenomic cosmid library generated from YGO6110. These cosmids were isolated and purified. Initial restriction analysis with rare-cutting enzymes indicated that they contained the CpG island of *Gabrb3*. Eight additional cosmids were identified with the α5 probe 300/301. Sixteen other clones were isolated from the YAC sublibrary by hybridization with the mouse

B1-B2 repetitive DNA. B1/B2 elements are short repetitive sequences dispersed throughout the mouse genome at a frequency of about 1 in every 10 kb (Krayev et al., 1982). Hybridization of these elements to the YGO713 cosmid library permits the differentiation of clones containing mouse segments from the clones that contain only yeast sequences. This is necessary since the YAC has not been purified from the other yeast chromosomes prior to subcloning.

Earlier attempts to isolate genomic clones containing the *Gabrb3* gene from cosmid and cDNA libraries were unsuccessful. In this cosmid library from YGO6110 only four clones were isolated in contrast to the eight clones recovered by hybridization with the *Gabra5* probe and sixteen clones containing mouse sequences (detected by hybridization to mouse repetitive DNA). It appears that the genomic region around *Gabrb3* may have have some structural feature that renders it difficult to clone in cosmids. About 8.9% of the genome is believed to have sequences that make them difficult to clone (Wyman et al., 1985).

Discussion

Physical mapping of the human *GABRA5-GABRB3* cluster (within 15q11-q13) has been extensively described because this region was initially thought to be associated with two distinct neurological syndromes, Prader-Willi (PWS) and Angelman's (AS) (Woodage et al., 1994; Sinnett, et al., 1993; Mutirangura et al., 1993; Kirkilionis et al., 1991). Although recent work has already excluded *GABRA5* and *GABRB3* from the chromosomal regions defined to contain genes responsible for the critical clinical phenotypes of PWS and AS (Reis et al., 1993; Sinnett et al., 1993), these studies have led to the construction of a high-resolution, long-range restriction map around these loci using field-inversion electrophoresis and phage genomic library screening of DNA from lymphocytes and lymphoblastoid cells (Sinnett et al., 1993; Woodage et al., 1994). Comparison of the murine mapping data presented in this study with the reported human physical map shows an extraordinary degree of conservation of the genomic structure and organization of these loci. The ~100-kb distance between 5' ends of the two genes, "head-to-head" transcriptional orientation, and the association with strong CpG islands at both the 5' ends of *Gabra5* and *Gabrb3* are identical in both man and mouse. Moreover, a CpG island was found to be located at the 3' end of *GABRA5* (Sinnett et al., 1993), and an island was also found in a similar location the homologous mouse region. This corresponds to the island closest to the right end of YGO713 (**Fig. 6**). A high degree of conservation of coding regions (Ymer et al., 1989; Wagstaff et al., 1991)

and 5' regulatory sequences (Kirkness and Fraser, 1993) between $GABA_A$ receptor subunit genes in mouse and man have also been reported. These data, along with the physical map described in this study showing similarity in overall genomic organization, argue for conservation of function of these genes, supporting our previous suggestion that 15q11-q13 be evaluated for possible role of *Gabrb3* in human cleft palate disorders (Culiat et al., 1993, 1994).

Physical distances of genetic markers extending distally from the *p* locus (**Fig. 11**) point out that the *p* deletions used for genetic analysis of *cp1* are relatively large deletions. For instance, $p^{4THO-II}$ deletes at least 1.4- 2.0 Mb of DNA. It is surprising that deletion of the region from *p* to *Gabra5*, which measures at least 1.2 Mb, does not yield any severe aberrant phenotypes (Culiat et al., 1994).

Whereas the 100-kb distance separating the 5' ends of *Gabra5* and *Gabrb3* is certainly large enough to contain other genes, it is small enough to reinforce the candidacy of *Gabrb3* as *cp1*. The $GABA_A$ receptor subunit genes have relatively short coding regions spread out in large genomic intervals. For example, the human *GABRB3* gene spans 250 kb, whereas *GABRA5* covers 70 kb (Sinnett et al., 1993). *GABRB1* has 14 kb of exons scattered throughout a 65-kb segment of genomic DNA (Kirkness et al., 1991). The mouse *Gabra5* and *Gabrb3* genes are organized similarly to their human counterparts, as indicated by the positions of 5' and 3' probes for each gene on the regional physical map (**Fig. 10**). *Gabrb3* is distributed over an area at least 100 kb, whereas the ~ 1.2-kb coding region of *Gabra5* is spread out into an area of at least 100

kb to at most ~150 kb. A 100-kb distance between *Gabra5* and *Gabrb3* may still contain one or two $GABA_A$ receptor subunit genes. However, it must be noted that, like the map of 15q11-q13, there are no other close clusters of rarecutter sites between murine *Gabra5* and *Gabrb3* (Sinnett et al., 1993 and **Fig. 11**). Since *Gabrg3* is also associated with a strong island (Stubbs, unpublished data), all the known genes in the $GABA_A$ receptor subunit cluster distal to *p* have a strong CpG island at their 5' ends. It is thus, very likely, although not certain, that the presence of another subunit gene would be reflected by the presence of another strong island. This does not rule out presence of another type of gene that is not associated with a CpG island or that shares the islands of either *Gabra5* or *Gabrb3*. Such a gene might account for the growth retardation phenotype manifested by $p^{4THO\text{-}II}$ homozygotes (Culiat et al., 1994; **Section V**.). If there are no other coding sequences in the interval, the growth retardation defect may simply be a consequence of any aberrant expression of the *Gabrb3* gene resulting from the absence of coding regions in p^{cp} and $p^{4THO\text{-}II}$ escapers (Nakatsu et al., 1993; Culiat et al., 1993, 1994) or the lower-than-wildtype expression of *Gabrb3* in $p^{4THO\text{-}II}$ homozygotes that have been rescued from the lethal cleft palate (**Section V**.).

Further molecular analysis of the sequences between *Gabra5* and *Gabrb3* can be accomplished by characterizing lambda, cosmid or P1 clones spanning the interval to search for known or unknown genes. Reagents are already available to initiate these experiments. For example, α5 and β3 cosmid subclones have been isolated from the cosmid library

generated from YGO713 (see section on Results). Glatt et al., (1994) are currently examining two overlapping human lambda clones, 12B-85 and 16B-86, that cover 23 kb midway between *GABRA5* and *GABRB3*. P1 clones can also be isolated with the 5' probes for both *Gabra5* and *Gabrb3*. This approach is promising because the bacteriophage P1 cloning system allows analysis of cloned DNA fragments as large as 75-100 kb (Sternberg, 1990), so that 1-2 clones can potentially span the *cp1* region. Genomic subclones of these long clones can be used directly as probes on "zoo blots" to search for conserved sequences among various species (eg. Monaco et al., 1986; Rommens et al., 1989) or to Northern blots and cDNA libraries. Alternatively, the clones could be used in direct selection (Lovett, Kere and Hinton, 1991) or exon amplification techniques (Buckler et al., 1991) to identify genes in the region. In direct selection, genomic clones, such as a YAC containing the interval where the gene of interest is located (eg. YGO713), can be immobilized in a filter and hybridized with a library of cDNA from tissue where the gene is likely to be expressed (eg. palate shelves at E14.5-E17.5). Non-specific hybridization is washed off and the bound cDNAs are eluted and amplified by PCR. The pool of eluted cDNAs can also be subjected to a second round of selection or cloned and characterized. In the exon amplication technique (Buckler et al., 1991), pieces of genomic clones containing the gene of interest are subcloned into a mammalian expression vector that allows identification of coding sequences that become cloned into the vector. The RNA transcribed from the exons are reverse transcribed and then amplified by PCR. The resulting products are cloned and analyzed.

Searching for other genes within the *cp1* interval is facilitated by the presence of the *p* deletion complex and the established restriction map of YGO713. Any potential candidate gene derived from direct selection or exon amplification can be used as probes hybridized to the deletion panel and YAC restriction digest blots to confirm its position. This will immediately rule out clones from direct selection that are retrieved by non-specific binding. The sequences of alternative candidate clones for *cp1* or for growth retardation phenotype can be scanned for homologies with known genes in computer databanks and the predicted amino acid sequence can be examined for structural motifs that might give clues to the function of the candidate gene.

SECTION IV.

FIGURES

FIGURE 1. DNA Analysis Showing that the Distal Breakpoint of *p*^{83FBFo} is within the *Gabra5* Gene and that *Gabra5* is Transcribed from a Distal to Proximal Direction Relative to *p*.

(**A**) A Southern blot of *Pvu*II-digested DNA, prepared from animals of the indicated genotype, was hybridized to a PCR-derived probe (385/386) for the 5' region of the *Gabra5* cDNA . This probe detects a 4.5-kb wild-type *Pvu*II fragment. The presence or absence of the fragment in the various genotypes indicated whether a particular *p* deletion removed this fragment of *Gabra5.* The hybridization pattern observed was the same as that observed for the 3' α5 probe except for the mutation *p*^{83FBFo}, which does not extend into the 5' region of *Gabra5* as defined by probe 385/386. Since *p*^{83FBFo} deletes the 3' coding region *of Gabra5* (Culiat et al., 1993) this indicated that: i) the distal breakpoint of *p*^{83FBFo} is within the *Gabra5* gene; and ii) *Gabra5* is transcribed fom a distal to proximal direction, in a head-to-head orientation with *Gabrb3.* A 0.7-kb fragment detected by a probe for the *Znf127 (D15S9h-1)* locus served as an indication of the amount of DNA loaded in each lane (lower panel of **Fig 1A**). (**B**) A map of chromosome 7 showing the transcriptional orientation of *Gabra5* and *Gabrb3* and the distal breakpoint of *p*^{83FBFo} at the *Gabra5* gene. The boxes represent the transcriptional units of *Gabrb3* and *Gabra5*. Since *Gabra5* has been excluded from being *cpl* (Culiat et al., 1993, 1994), the interval containing *cpl* must be immediately distal to this gene. The physical distance separating *Gabra5* and *Gabrb3* must therefore be the maximum size of the area where *cpl* is located.

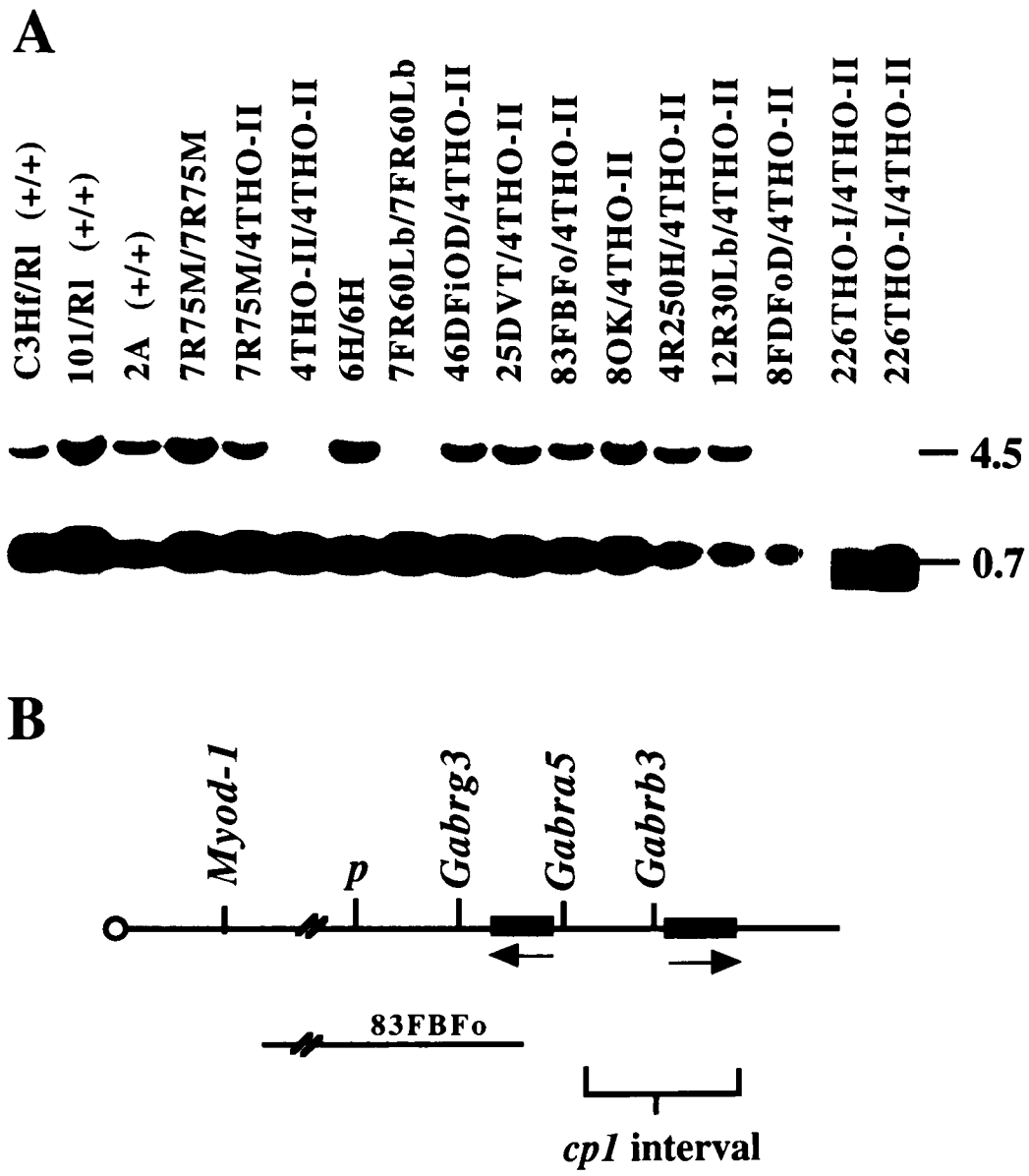
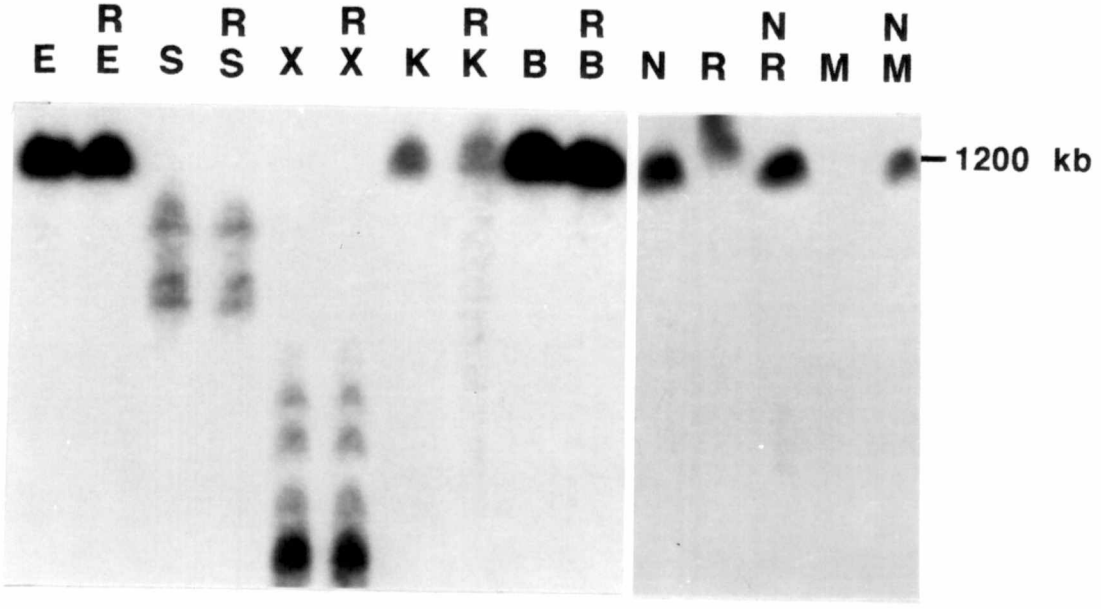

A
C3Hf/Rl (+/+)
101/Rl (+/+)
2A (+/+)
7R75M/7R75M
7R75M/4THO-II
4THO-II/4THO-II
6H/6H
7FR60Lb/7FR60Lb
46DFiOD/4THO-II
25DVT/4THO-II
83FBFo/4THO-II
8OK/4THO-II
4R250H/4THO-II
12R30Lb/4THO-II
8FDFoD/4THO-II
226THO-I/4THO-II
226THO-I/4THO-II
4.5
0.7
B
Myod-1
p
Gabrg3
Gabra5
Gabrb3
83FBFo
cp1 interval

FIGURE 2. Hybridizations of *Gabrb3* (218/219; 3') and *Gabra5* (300/301; 3') Probes to PFGE Blots

(Upper panel): The *Gabrb3* probe detects strong signals for ~1200-kb fragments in *Eag*I, *Ksp*I, *BssH*II, and *Not*I digests. Double digests with *Nru*I and *Not*I also generate the same size fragment. (Lower panel): The α5 probe, 300/301 (3' *Gabra5*), is located in small PFGE fragments (220-300 kb). Unlike 218/219 (3' *Gabrb3*), the α5 probe does not pick up strong signals. There are no fragments to which probes 218/219 and 300/301 co-hybridize. Restriction enzymes used to digest mouse spleen DNA into large genomic fragments are designated as: *Eag*I (E), *Nru*I (R), *Sal*I (S), *Xho*I (X), *Ksp*I (K), *BssH*II (B), *Not*I(N), *Mlu*I (M), *Sfi*I (F) , *Cla*I (C), *SmaI* (Sm), *BstbI* (Bt).

β3


E
R E
S
R S
X
R X
K
R K
B
R B
N
R
N R
M
N M
1200 kb

α5

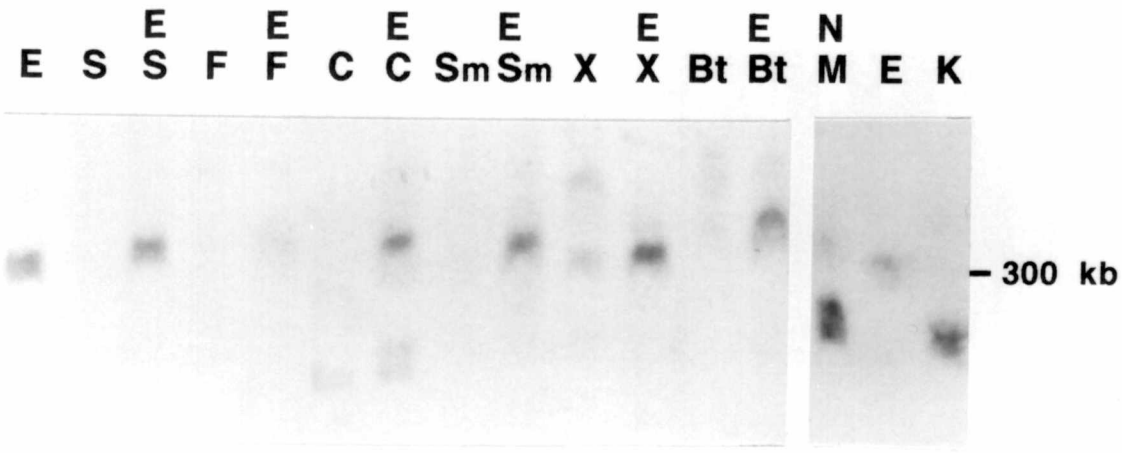

E
S
E S
F
E F
C
E C
Sm
E Sm
X
E X
Bt
E Bt
N M
E
K
300 kb

FIGURE 3. Identification of YACS Spanning the Region *Gabra5* to *Gabrb3*

A Southern blot of *Eco*RI digests of YACS YGO713, YGO813, YBO392 and YGO6110 was hybridized with 5' β3 (280/281) and 3' α5 probes (300/301). Only YGO713 / YGO813 contain *Gabra5* sequences, as indicated by hybridization of the 3' α5 probe to a 3.0-kb genomic fragment. All the YACs have the 5' region of *Gabrb3*. Both YG06110 and YBO392 have all three wild-type genomic DNA fragments (23, 9.5 and 8.0 kb) that hybridize to 280/281, while the α5 YACS, YGO713/YGO813, contain only the 23-kb fragment. Since YGO713/YGO813 has *Gabra5* and part of *Gabrb3*, the *cp1* interval is localized within this ~430 kb YAC.

3'α 5
C3Hf (+/+)
GO813
GO713
GO6110
BO392
— 3.0
5'β 3
C3Hf (+/+)
GO813
GO713
GO6110
BO392
— 23.0
— 9.5
— 8.0

FIGURE 4. Mapping of the Right Ends of YGO6110 and YGO713 Relative to the Distal Breakpoints of *p* Deletions.

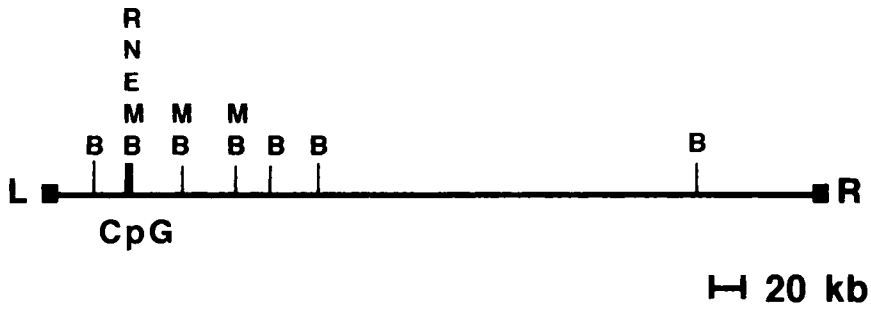
A) The right endclone of the YAC YG06110 was hybridized to a Southern blot of *Bgl*II-digested DNA from various *p*-deletion mutants having the genotypes indicated above the blot. This end was deleted in $p^{4THO\text{-}II}$ and $p^{7FR60Lb}$ homozygotes. The probe recognizes a sequence present in the other deletions. This hybridization pattern is consistent with the right end of YG06110 being distal to the breakpoints of these other deletions. Since the most distal of these ends is that of p^{8FDFoD}, which does not extend beyond *Gabrb3*, the right end must be between *Gabrb3* and *Znf127 (D15S9h-1)*.

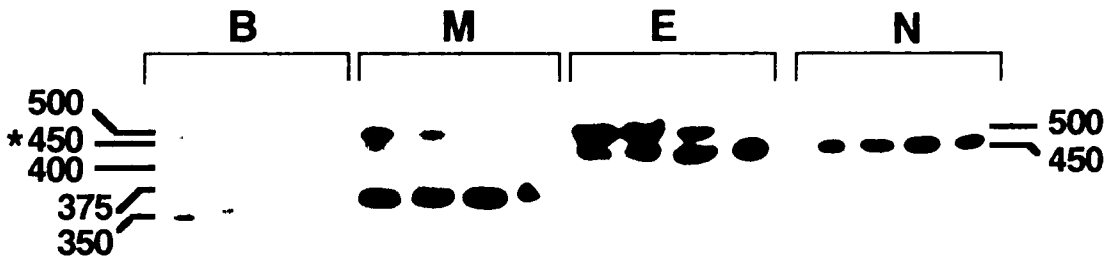
B) Hybridization of the right endclone of YGO713 to a Southern blot of *Pvu*II-digested DNAs with the same genotypes (identical order in the blot used in **A**) indicated the absence of the fragment in $p^{4THO\text{-}II}$ (lane F) and $p^{7FR60Lb}$ homozygotes (lane **H**) and also in $p^{83FBF0}/p^{4THO\text{-}II}$ DNA (lane **F**). The endclone is not deleted in the other *p* deletions tested, and the data are consistent with the position of this end towards *p*.

A
C3Hf/RI (+/+)
101/RI (+/+)
2A (+/+)
7R75M/7R75M
7R75M/4THO-II
4THO-II/4THO-II
6H/6H
7FR60Lb/7FR60Lb
46DFiOD/4THO-II
25DVT/4THO-II
83FBFo/4THO-II
80K/4THO-II
4R250H/4THO-II
12R30Lb/4THO-II
8FDFoD/4THO-II
B
A B C D E F G H I J K L M N O

FIGURE 5. Restriction Map of YGO6110

The position of rare-cutting enzymes *BssH*II (*B*), *Eag*I(*E*), *Mlu*I (*M*), *Not*I *(N)* and *Nru*I (*R*) in YGO6110 reveal the presence of a strong CpG island ~ 50 bases from the left end (top panel). A series of *Bss*HII sites about 25-50 kb apart are located at the left end. YGO6110 is oriented such that the left end is towards *p* and the right end is towards *D15S9h-1 (Znf127).* This map was based on hybridization of right (middle panel) and left (bottom panel) endclones of YGO6110 to blots containing partially digested YAC DNA. The four lanes under each enzyme represent (from left to right) increasing degree of digestion.


R
N
E
M M M
B B B B B B
B
L
R
CpG
20 kb


B
M
E
N
500
*450
400
375
350
500
450


75

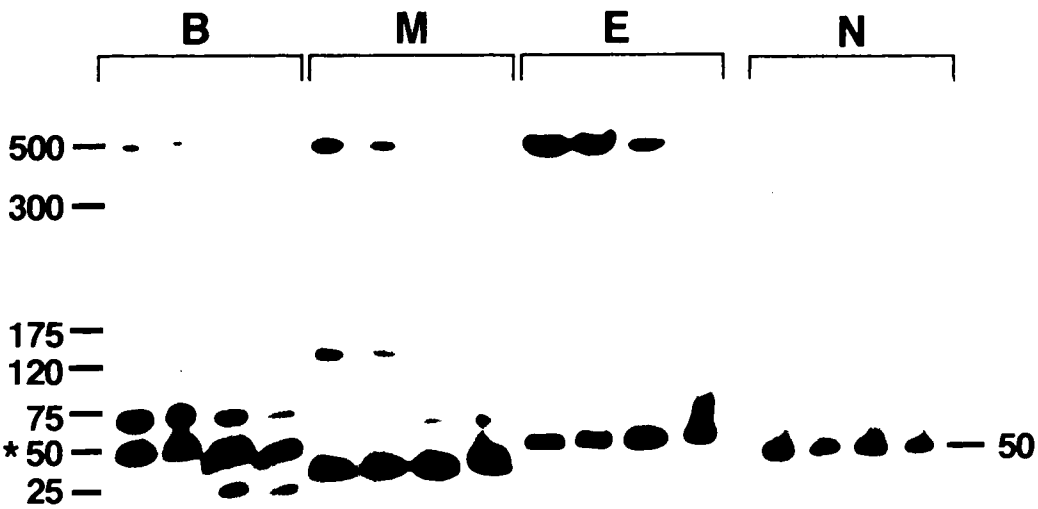
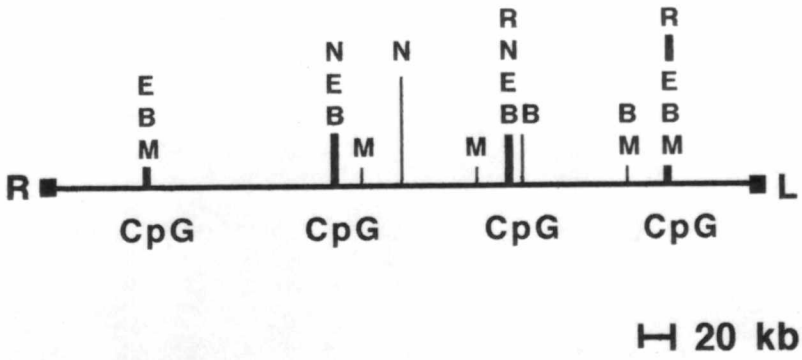
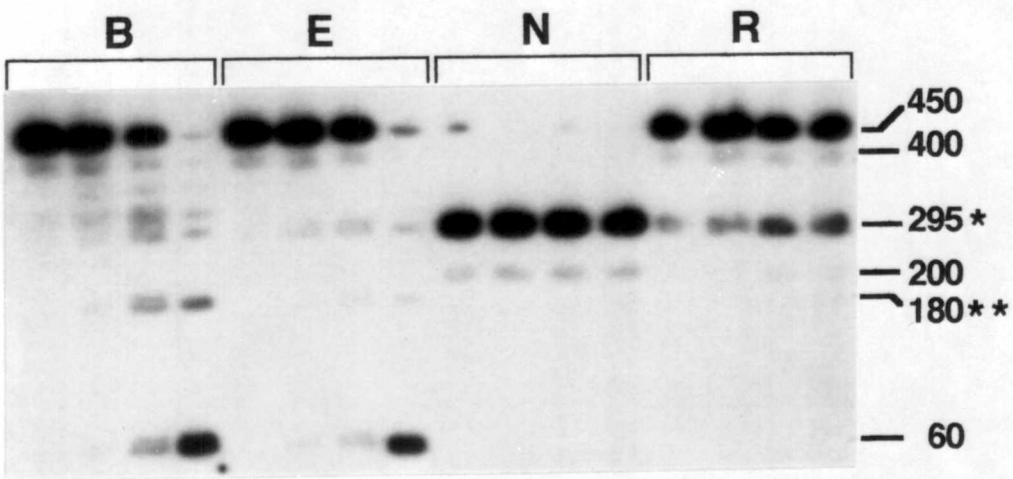

B
M
E
N
500
300
175
120
75
*50
25
50

FIGURE 6. Restriction Map of YGO713

The positions of rare-cutting enzymes *Bss*HII (*B*), *Eag*I (*E*), *Mlu*I (*M*), *Not*I (*N*) and *Nru*I (*R*) in YGO713 indicate the presence of four CpG islands (top panel). YGO713 is oriented such that right end is towards *p* and the left end is towards *D15S9h-1 (Znf127)*. Hybridization of the right endclone to blots with partially digested DNA is shown in the middle and bottom panels. The four lanes under each enzyme represent (from left to right) increasing degree of digestion. Further analyses identified the islands located ~180 kb (**) and ~295 kb (*) from the right arm (**R**) of the YGO713 are at the 5' regions of *Gabra5* and *Gabrb3*, respectively. This narrowed down the region where *cpl* is located (the distance of α5 and β3) to a 100-kb interval.


R
L
E
B
M
N
E
B
M
N
M
R
N
E
B
B
B
M
R
E
B
M
CpG
CpG
CpG
CpG
20 kb


B
E
N
R
450
400
295 *
200
180 **
60

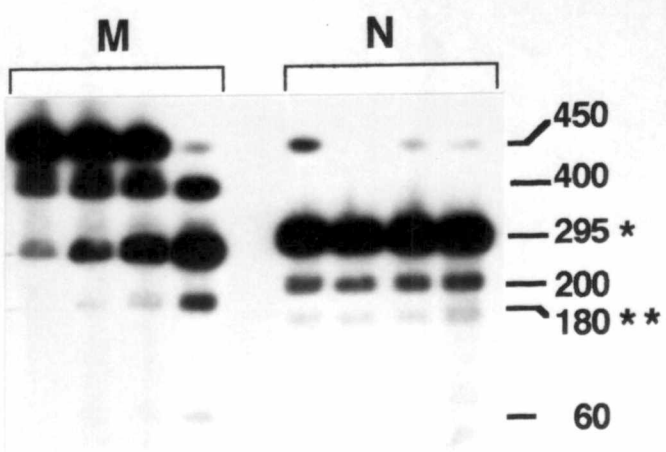
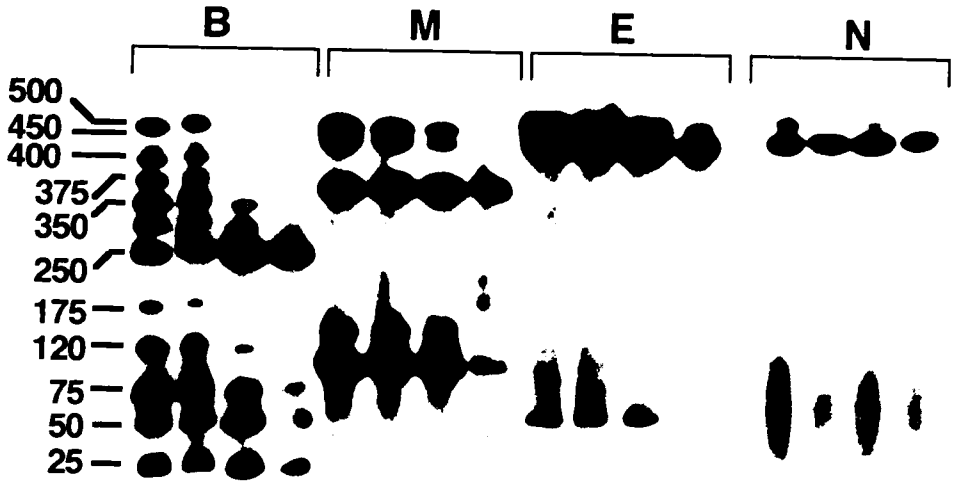

M
N
450
400
295 *
200
180 **
60

FIGURE 7. Mapping of the 5' and 3' Probes of *Gabrb3* in YGO6110

The probes for the 3' (218/219) and 5' (280/281) regions of *Gabrb3* were hybridized to the same Southern blot containing partial digests of YG06110 by the following enzymes: (B) *Bss*HII, (M) *Mlu*I, (E) *Eag*I, and (N) *Not*I. The hybridization of the 5' probe with numerous small *Bss*HII and *Mlu*I fragments and the larger *Not*I fragment is consistent with its placement near the strong CpG island in YGO6110 (**Fig. 5**). Association of the 5' *Gabrb3* probe (280/281) with this island agrees with the prior pulsed-field gel electrophoresis data and hybridization of the probe to *Bam*HI double digests. The DNA analysis shown in the lower panel maps the 3' *Gabrb3* probe between the first and and second *Bss*HII sites from the right end of the YGO6110. For instance, it does not detect the small *Mlu*I and *Bss*HII fragments towards the left end of the YAC (**Fig. 5**). The distance between the two probes is therefore from 100 to 300 kb (from CpG island to first *Bss*HII site from right end).

5' β3 (280/281)



3' β3 (218/219)

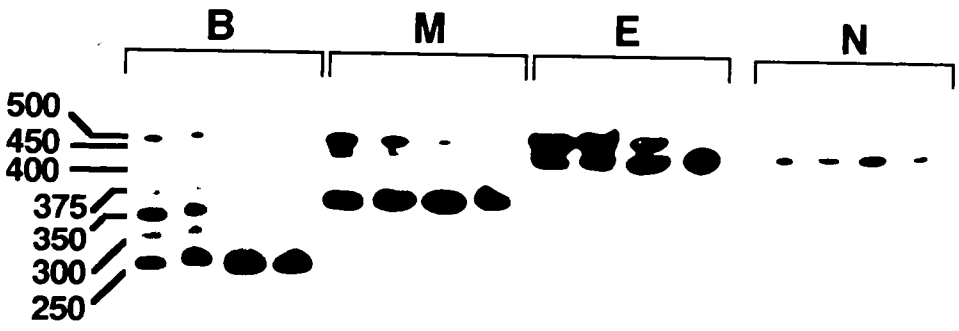
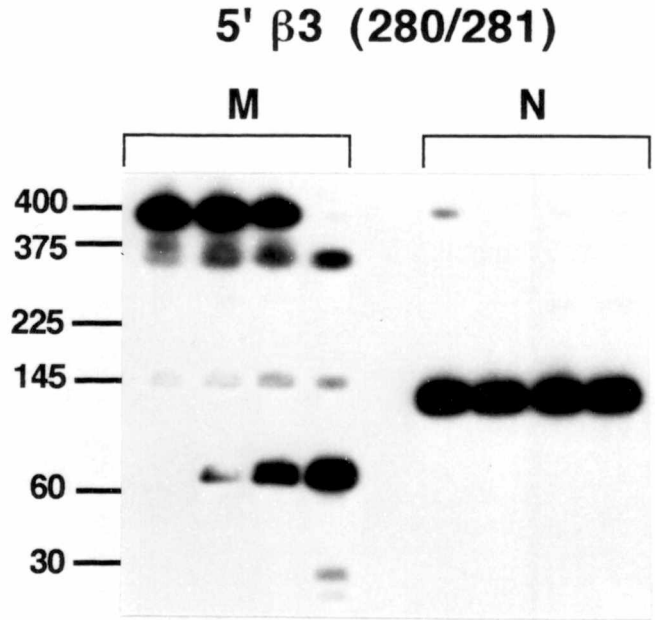


FIGURE 8. Mapping *Gabrb3* and Establishing Restriction Sites Relative to the Left End of YGO713

Probe 280/281 (*5' Gabrb3*) and the left endclone of YGO713 were hybridized to a blot with *Mlu*I (M) and *Not*I (N) partial digests of YGO713. Hybridization with *Bss*HII, *Eag*I partial digests were also performed but the data are not shown here. The hybridization pattern of the probe for the 5' coding region of *Gabrb3* links it with a CpG island ~150 kb away from the left end (**Fig. 6**). *Mlu*I and *Not*I sites detected by hybridization of the left endclone are identical to those determined by hybridization with the right end.


5' β3 (280/281)
M
N
400
375
225
145
60
30

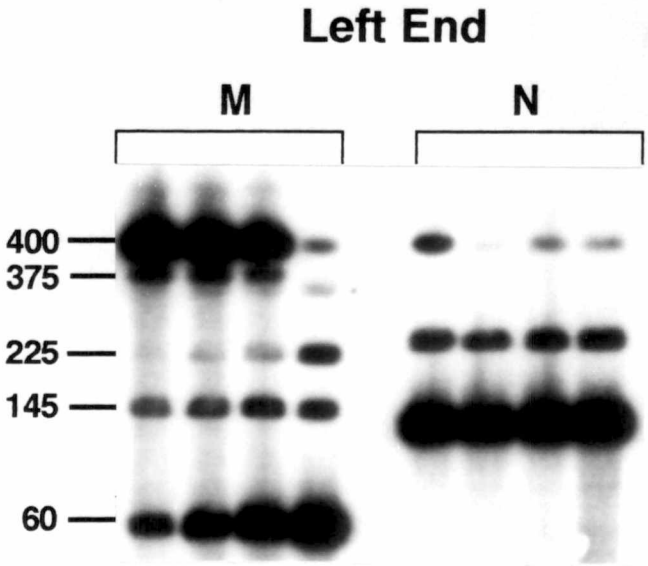

Left End
M
N
400
375
225
145
60

FIGURE 9. Mapping of 5' and 3' Regions of *Gabra5* in YG0713

A probe corresponding to a sequence towards the 3' region of *Gabra5* (300/301) detected fragments in *Mlu*I and *Not*I partial digests of YGO713 identical to those detected by a probe for right end of the YAC, thereby positioning this portion of the gene very near the right end, proximal to *p* (**Fig. 6**). Hybridization of a probe for the 5' end of *Gabra5* (385/386) indicates association with the CpG island located 180-200 kb from the right end.

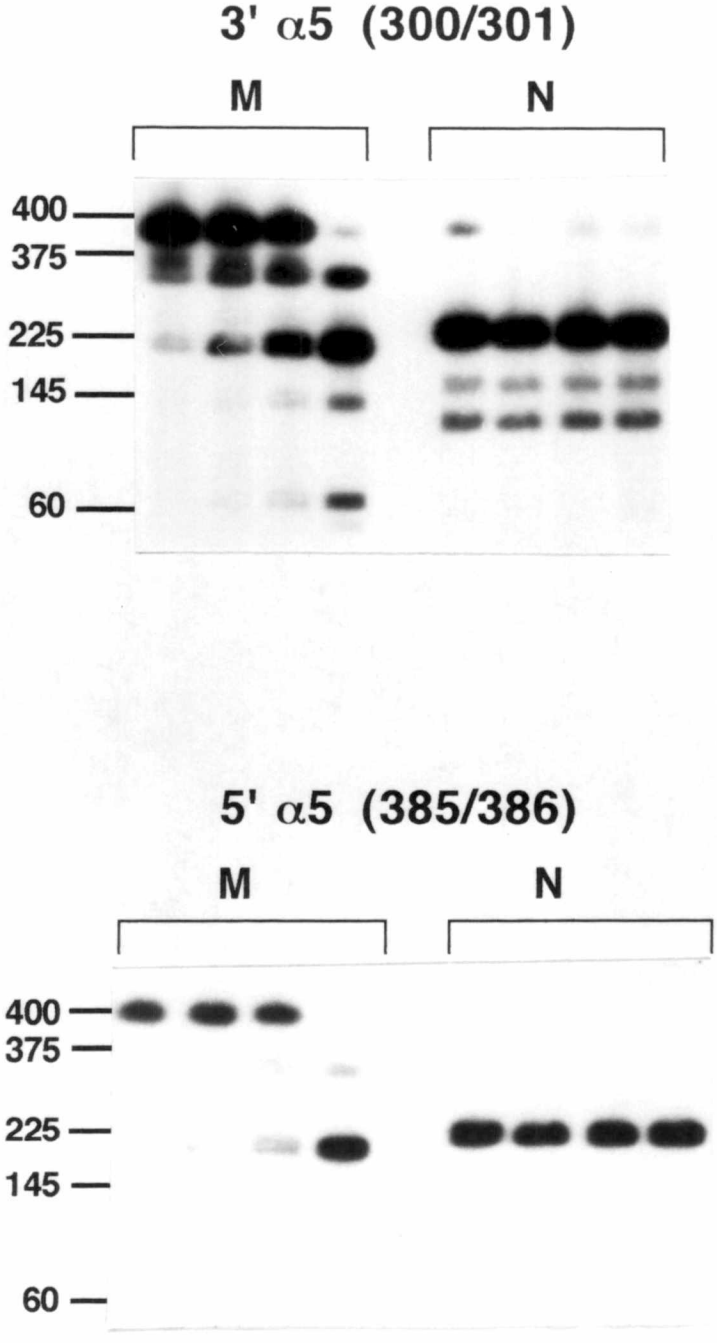
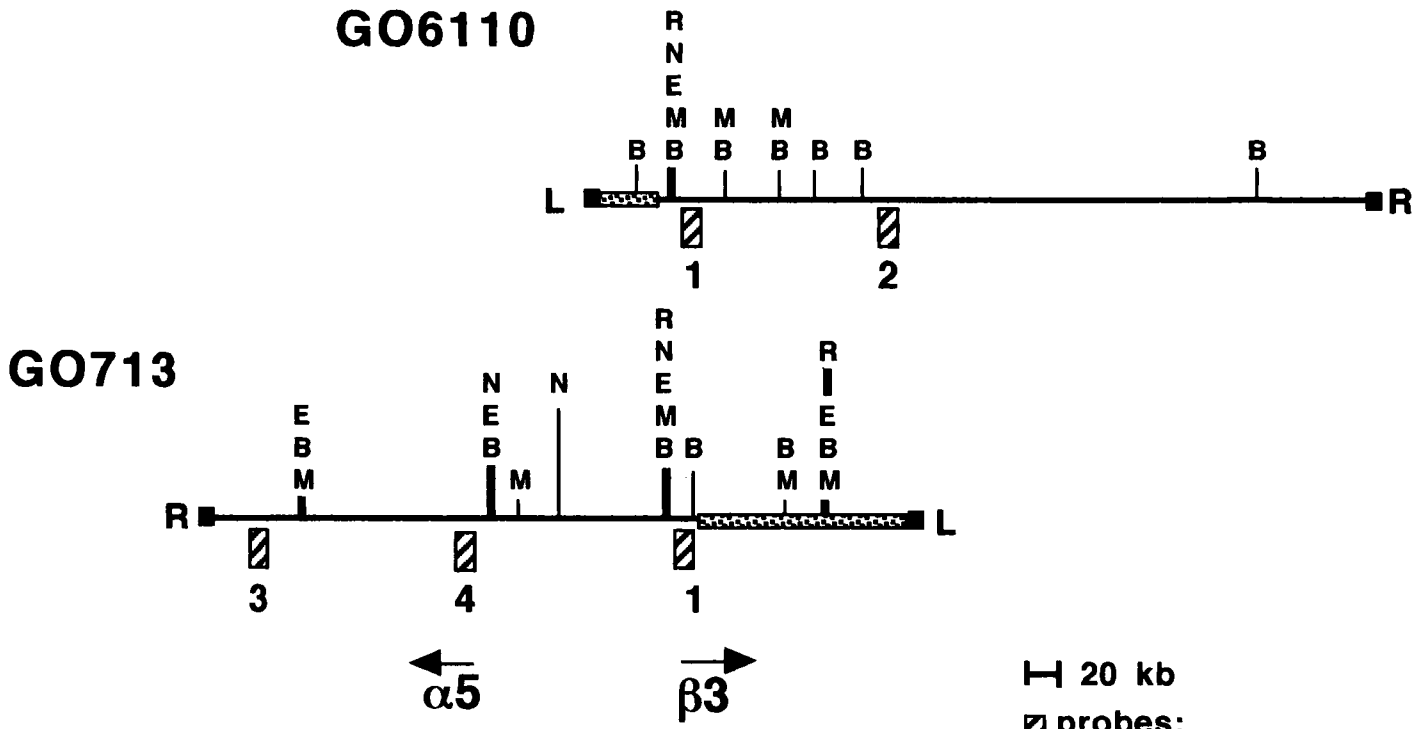

3' α5 (300/301)
M
N
400
375
225
145
60
5' α5 (385/386)
M
N
400
375
225
145
60

FIGURE 10. Overlap of Restriction Maps of YACS Spanning *cp1* and Position of Probes for *Gabra5* and *Gabrb3*

The overlap of YGO6110 and YGO713 at their left ends include the *Gabrb3* island. A comparison of the restriction maps suggests that the left end of YGO713 is probably chimeric because of the absence of the series of *Bss*HII sites and the presence of a strong CpG island 100 kb distal to *Gabrb3,* which is not present in YGO6110. The PFGE analysis of wildtype genomic DNA also does not show the presence of this particular CpG island. Positions of the 5' and 3' PCR probes for *Gabra5* and *Gabrb3* show that these genes are spread out over a large genomic distance.



20 kb

probes:

1 280/281(5'β3)

2 218/219(3'β3)

3 300/301(3'α5)

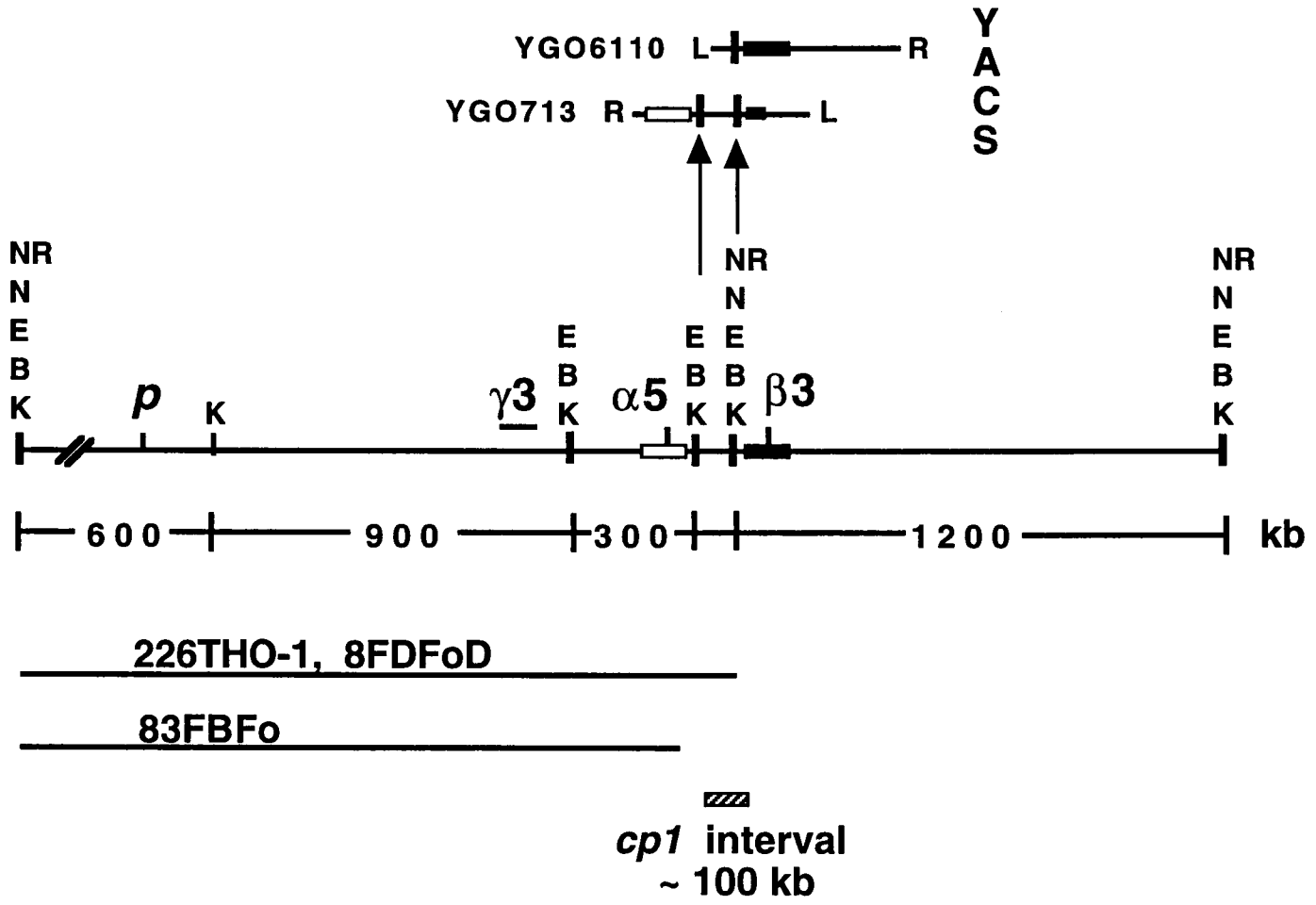
4 385/386(5' α5)

chimeric ends

FIGURE 11. The Physical Map of the *Gabrg3-Gabra5-Gabrb3* Gene Cluster Distal to the *p* Locus on Mouse Chromosome 7 Suggests that the *cp1* Interval is ~100 kb in Length

Genetic and molecular methods localized the *cp1* gene between the distal breakpoints of p^{83FBFo} and $p^{226THO\text{-}II}$ or p^{8FDFoD}. Because these distal endpoints are at the coding region of *Gabra5* and the 5' region of *Gabrb3*, respectively, the distance between these genes defines the length of the *cp1* interval. Integration of the molecular analysis and restriction maps of YGO713 and YGO6110 with PFGE data of the region *p-Gabrg3-Gabra5-Gabrb3* is consistent with the association of *Gabra5* and *Gabrb3* with strong CpG islands separated by ~100 kb.

Restriction enzymes are designated as *Bss*HII (*B*), *Eag*I (*E*), *Ksp*I (*K*), *Not*I (*N*), *Nru*I (*NR*). R and L correspond to the right and left ends of the YACs. The solid black boxes represent the extent of the *Gabrb3* gene in the YACs and in mouse chromosome 7, whereas the white boxes represent the extent of *Gabra5*. Preliminary data link *Gabrg3* (γ3) to the island proximal to *Gabra5*.


YGO6110 L R
YGO713 R L
YACS
NR
NEBK
p
K
γ3
EBK
α5
EBK
NR
NEBK
β3
NR
NEBK
600
900
300
1200
kb
226THO-1, 8FDFoD
83FBFo
cp1 interval
~ 100 kb

V. RESCUE OF $p^{4THO-II}$ / $p^{4THO-II}$ MICE FROM LETHAL CLEFT PALATE BY EXPRESSION OF A *Gabrb3* TRANSGENE

Introduction

The direct causal relationship between the activity of a particular gene and a specific phenotype is usually established rigorously either by mutating the candidate gene and generating the corresponding mutant phenotype or by correcting the defect in mutants by expressing the candidate gene. In the first approach, targeted mutagenesis of a gene of interest is accomplished by homologous recombination between the endogenous gene and a portion of the gene cloned in a targeting vector, along with a selectable marker. This recombination is designed in such a way that the endogenous gene is disrupted, creating a null mutation. In the second approach, the candidate gene (or cDNA) is introduced into the genome of mouse mutants using standard techniques for generating transgenic mice. The phenotype-rescue approach was chosen for this project because we did not have access to cloned and characterized genomic fragments of mouse *Gabrb3* that could have been used for homologous recombination techniques to "knock out" the gene. Hybridization of the *Gabrb3* probe, 218/219, to mouse cosmid and cDNA libraries failed to identify genomic clones that could be used for gene "knock-out" experiments. Moreover, this technology was just being set-

up at the Biology Division of Oak Ridge National Laboratory at the time this decision had to be made.

Figs. 1 and **2** summarize the construction of the transgene used for the rescue experiment and **Fig. 3** outlines the genetic scheme used to correct the CP phenotype in *p4THO-II/p4THO-II* mutants. Six transgenic founder animals were obtained from two separate pronuclear microinjection experiments. Data on the genotypes and transmission frequencies of all founders are summarized in **Table 1.** Most of the data presented here are from experiments on the first founder line, *TgN(Gabrb3)5023Rn.* A summary of the recovery of rescued mice is presented in **Table 2.**

The following section describes the rescue experiment and will be submitted to *Science* for publication (Culiat et al., in preparation). This manuscript is entitled "**Deficiency of the β3 Subunit of the Type-A γ-aminobutyric Acid Receptor Causes Cleft Palate in Mice**". The manuscript describes the phenotype-rescue experiment that showed correction of both the lethal cleft palate as well as the neurological defects manifested by rare "escapers" observed in mice homozygous for the *p4THO-II* mutation. These data suggest the dual role of β3 subunit of the $GABA_A$ receptor in both palate development and neuronal function. Speculation about the possibility of neurotransmitters acting as factors for specific differentiation (hormone-like) events (eg. palatogenesis) is also presented.

Submitted to: *Science*

Deficiency of the β3 Subunit of the Type A γ-aminobutyric Acid Receptor Causes Cleft Palate in Mice

Cymbeline T. Culiat[§], Lisa J. Stubbs, Richard P. Woychik, Liane B. Russell, Dabney K. Johnson, and Eugene M. Rinchik

Biology Division and [§]University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge National Laboratory, P.O. Box 2009, Oak Ridge, TN 37831-8077 (U.S.A.),

Offprint requests to : E. M. Rinchik. Present address:

Sarah Lawrence College
1 Mead Way
Bronxville, NY 10708

Abstract

Binding of γ-aminobutyric acid (GABA) to the type-A GABA ($GABA_A$) receptor results in the opening of an integral membrane chloride channel in neurons, thereby mediating neuronal inhibition. In addition to its function in the nervous system, GABA has been implicated in mouse craniofacial development by the results of several teratological, and more recently, genetic studies. Here we provide direct evidence that *Gabrb3*, the gene coding for the β3 subunit polypeptide of the $GABA_A$ receptor, is identical to *cpl* (cleft palate-1), a gene(s) previously mapped to chromosome 7 and shown to be necessary for secondary palate development in mice. Cleft palate in mice homozygous for a chromosome 7 deletion that includes the *Gabrb3* locus was corrected by the introduction of a rat *Gabrb3* cDNA transgene under the control of the human β-actin promoter. The rescued deletion homozygotes are typically smaller than their littermates, but this decrease in size correlates with homozygosity for the deletion rather than with expression of the transgene. No detectable aberrant phenotypes could be attributed to transgene expression, despite the fact that the transgene was active in tissues where *Gabrb3* is not normally expressed. These data strongly suggest that, in addition to the well-documented function of GABA receptors in the nervous system, at least one neurotransmitter receptor polypeptide is involved in craniofacial development in mammals. The homologous gene, *GABRB3*, in human chromosome 15q11-q13 near the Prader-Willi and Angelman syndrome regions, should therefore be

evaluated in the context of inherited or sporadic craniofacial disorders in humans.

Introduction

Facial clefting is a prevalent birth defect in the human population, exhibiting an incidence of ~1/1000 live births with little racial variation (Moore et al., 1991). Clefting of the secondary palate (roof of the mouth) usually occurs in conjunction with clefting of the lip (CL/P) and other syndromic defects; however, isolated cleft palate (CP) is genetically and developmentally distinct from CL/P (Fogh-Andersen, 1942). The patterns of inheritance exhibited by facial clefting are so complex that determining the genetic component of this phenotype has been difficult, and numerous hypotheses on how facial clefting might be inherited have been proposed (Fraser, 1989). Although the majority of cases may be due to the interaction of various genes in conjunction with environmental factors (i.e., a multifactorial model), at least one major locus in the Xq13-q21 region of human X chromosome has been associated with CP (Stanier et al., 1993).

The mouse is an excellent model system for identifying genetic factors that govern the complicated process of craniofacial development because a number of mutations in the mouse result in facial anomalies either as a primary phenotype or as a component of a more complex syndrome (Green, 1989). In mice, CP of genetic origin is generally attributed to a failure of the palate shelves to elevate and fuse (Johnston

and Bronsky, 1991; Flint, 1980). At least three major mouse loci have been correlated with this type of defect: the type-II collagen gene on chr 12 (Francomano et al., 1987); a locus on chr 8 that controls susceptibility to glucocorticoid-induced CP (Karolyi et al., 1990); and *cp1* on chr 7 (Culiat et al., 1993). Recently, null mutations of the homeobox-containing gene *Msx1* and of the gene coding for transforming growth factor β3 (*TGFβ3*) were shown to generate cleft palate in mice (Satokata and Maas, 1994; Ferguson, 1994; Proetzel and Doestchman, 1994). The characterization of such mutant genes should provide major clues into the underlying mechanisms of both normal and abnormal palate formation in mammals.

Disruption of the *cp1* gene in $p^{4THO-II}$, a radiation-induced deletion mutation that includes the pink-eyed dilution (*p*) gene in mouse chromosome 7, results in recessive, neonatally lethal CP in mice homozygous for the deletion (Culiat et al., 1993). Genetic and molecular analyses of 19 additional *p* deletions localized *cp1* to a specific interval distal to *p*, between *Gabra5* and *Gabrb3*, the genes encoding the α5 and ß3 subunits of the $GABA_A$ receptor, respectively (Culiat et al., 1993). Complete concordance was observed between alterations in *Gabrb3* and inability to complement the clefting defect, and RNA expression data showed the presence of *Gabrb3* mRNA in fetal heads at 14.5 days of gestation, the period critical for palate elevation (Culiat et al., 1993). These genetic data supported earlier teratological studies that implicated GABA and its agonists in the etiology of CP in mice (Wee and Zimmerman, 1983; Zimmerman and Wee, 1984; Zimmerman, 1985; Miller and Becker,

1975). For example, the GABA agonist diazepam is mildly teratogenic and induced cleft palate when administered to pregnant mice and rats (Miller and Becker, 1975; Wee and Zimmerman, 1983); GABA itself is found in embryonic palatal structures during critical stages of palate-shelf elevation and closure (Wee et al., 1986; Wee and Zimmerman, 1985); and GABA or diazepam inhibits palate-shelf elevation *in vitro* (Wee and Zimmerman, 1983; Zimmerman and Wee, 1984; Zimmerman, 1985). Based on all available data, we speculated (Culiat et al., 1993) that *Gabrb3* is *cp1* and that normal palate development requires the activity of a $GABA_A$ receptor that includes the β3 subunit polypeptide. We demonstrate here that a cDNA transgene encoding the rat *Gabrb3* gene corrects the CP defect in $p^{4THO-II}$ homozygotes, thereby providing direct proof that the ß3 subunit of the $GABA_A$ receptor is indeed required for normal palate formation in mice.

Materials and Methods

Mice

All mouse stocks used in this study were maintained and bred at the Mammalian Genetics Section, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN. The origin of $p^{4THO-II}$ has been reported (Culiat et al., 1993; Nicholls et al., 1993). p^{7R75M} is a mild allele of *p* that

has darker pigmentation when homozygous than when heterozygous with most alleles of *p*.

In the phenotype-rescue experiment, wild-type FVB/N females were mated to $p^{7R75M}/p^{4THO-II}$ males, and single-celled fertilized eggs were microinjected with the transgene. The surrogate mothers of the injected embryos were wild-type (C3Hf X 101)F_1 females that were plugged by vasectomized, wild-type FVB/N males. All surgical procedures such as harvesting of fertilized eggs, oviduct transfers, and vasectomy were performed according to the standard protocols (Hogan et al. 1986). Before any surgical procedure was performed, the anaesthetic avertin (1.5 g tribromoethanol, 0.93 ml isoamyl alcohol, and 118.5 ml water) was administered by an intraperitoneal injection of 0.024-0.028 ml /g body weight.

The six transgenic lines generated were named based on the *Standardized Nomenclature for Transgenic Animals*, published by the National Research Council (*ILAR News*, 1992). Names of the lines are as follows (written in bold are the shortened names used in labelling cages and entries in breeding cards):

a) *TgN(Gabrb3)5023Rn* (**1TGβ3**)

b) *TgN(Gabrb3)5047Rn* (**2TGβ3**)

c) *TgN(Gabrb3)5058Rn* (**3TGβ3**)

d) *TgN(Gabrb3)5071Rn* (**4TGβ3**)

e) *TgN(Gabrb3)5074Rn* (**5TGβ3**)

f) *TgN(Gabrb3)5115Rn* (**6TGβ3**)

Since transgenic founders can be either $+/p^{7R75M}$ or $+/p^{4THO-II}$, in order

to determine the genotype of a founder, it was mated to a $p^{7R75M}/p^{4THO\text{-}II}$ mouse. If a founder were $+/p^{7R75M}$, it will have p^{7R75M}/p^{7R75M} progeny, but a $+/p^{4THO\text{-}II}$ founder will yield pink-eyed pups ($p^{4THO\text{-}II}/p^{4THO\text{-}II}$) and no p^{7R75M}/p^{7R75M} progeny. These genotypes are easily detected by differences in eye- and coat-color pigmentation. If the founder were $+/p^{4THO\text{-}II}$, this mating could immediately produce the $p^{4THO\text{-}II}$ homozygotes in which rescue could be evaluated. In the case where the founder was $+/p^{7R75M}$, the progeny were screened for the presence of the transgene, and transgenic $p^{7R75M}/p^{4THO\text{-}II}$ mice were mated to non-transgenic $p^{7R75M}/p^{4THO\text{-}II}$ mice to generate transgenic $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ segregants. Expression of the transgene was evaluated in each transgenic founder line by RNA analysis of various tissues from an adult progeny derived from the founder.

All mice used in this study were handled in accordance with the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.*

DNA Analyses

DNA was isolated from ~1-inch-long tail biopsy cut from mice being screened for the presence of the transgene. The tails were digested in a buffer solution containing proteinase K (100 ng/μl) and were incubated overnight at 42 °C. The DNA was isolated by phenol extraction followed by precipitation in ethanol, and was finally resuspended in 400 μl TE

buffer (10 mM Tris pH 7.5, 1.0 mM EDTA).

Preparation of DNA and Southern-blot analyses were done by standard procedures (Sambrook et al., 1989). In southern blotting, 10 µg of each DNA sample were digested overnight in 50-µl reactions and electrophoresed in 0.8% agarose. DNA in the gel was nicked by exposure to UV at 120, 000 µjoules/m^2; denatured in alkali; neutralized then blotted onto Duralon nylon filters in 10 X SSC buffer. Filters were rinsed in 2X SSC, baked for 1-2 hours under vacuum and then immediately crosslinked with UV at 25, 000 µjoules/m^2.

Hybridizations of Southern blots with radiolabelled probes were carried out at 42 °C, overnight. Filters were washed at a stringency of 1.0 or 0.2 X standard saline/citrate/0.2% sodium dodecyl sulfate (SDS) (depending on the probe) at 68°C for 30 min.

RNA Analyses

Extraction of total RNA from E17.5 or E18.5 fetal heads and various adult mouse tissues by cesium chloride gradients and Northern blotting were done according to standard procedures (Sambrook et al.,, 1989). Poly-A^+ RNA was isolated from total RNA samples by affinity chromatography using commercial kits from Mini-Ribosep Oligo-dT columns (Collaborative Biomedical Products). In Northern blots having total RNA, 20 µg of sample were loaded for each lane, whereas 1-3 µg were used for samples of polyA^+ RNA. Sizes of the transcripts were

estimated by comparison to commercial RNA molecular weight markers (Life Technologies). The hybridization procedure was identical to that employed in DNA analysis. A probe for chick tubulin was hybridized to the filters as a loading control.

Construction of Transgene Ubiquitously Expressing Rat *Gabrb3*

An outline of the cloning strategy used to construct a transgene expressing a full-length rat *Gabrb3* cDNA is illustrated in **Fig. 1.** The rat *Gabrb3* cDNA clone used in this construct was kindly provided by D. B. Pritchett, Department of Neuroscience and Anatomy, Children's Hospital, Philadelphia, PA 19104. The cDNA was excised as an *Nru*I-*Bam*HI fragment from a vector previously used for expression in *Xenopus* oocytes (Ymer et al., 1989). The *Nru*I site is 48 bp upstream of the start codon. *Mlu*I linkers were attached to this fragment and then inserted to the *Mlu*I site of a vector, designated *pBAPBS*, which was derived from the plasmids *pBAP.2M* and a commercial Bluescript vector (*pBSSK*). *pBAP.2M* is a modification of a human β-actin expression vector (Gunning et al., 1987) produced by the addition of an *Mlu*I site to the polylinker region. This was necessary because all the enzymes recognizing the original cloning sites cut the *Gabrb3* cDNA. The 4.5-kb expression cassette of *pBAP.2M* is composed of the human actin promoter - 5' untranslated region - an intron-polylinker (*Mlu*I, *Hind*III, *Sal*I, and *Bam*HI)-SV40 polyadenylation site. The entire cassette was removed as a *Cla*I fragment and inserted into the

*Cla*I site of *pBSSK,* generating the final cloning vector for the cDNA, designated *pBAPβ3*. The latter step was necessary because *Cla1* also cuts the *Gabrb3* cDNA, and putting the cassette into the *pBSSK* cloning region allows it to be flanked by enzymes (e. g. *Xba*I and *Kpn*I) that will liberate the transgene construct intact. The 6.5-kb *Xba*I-*Kpn*I transgene fragment is designated *BAPβ3*.

Genetic Strategy for Rescuing $p^{4THO-II}/p^{4THO-II}$ Mice from Cleft Palate

Fig. 3 illustrates the breeding scheme to generate $p^{4THO-II}/p^{4THO-II}$ mice expressing a *Gabrb3* transgene. Superovulation of FVB females was achieved by administration of pregnant mares serum, 3 days prior to harvesting eggs. One day before harvest, females were injected with human chorionic gonadotrophin and mated to $p^{7R75M}/p^{4THO-II}$ males, overnight. Fertilized eggs were isolated from plugged females and incubated in M16 medium at 37 ^{o}C for 3-5 hours. One-celled fertilized eggs were transferred to M2 medium and the male pronuclei were injected with the transgene DNA (5 ng/μl concentration). The cells were cultured overnight in M16 medium. Preparation of hormones, culture media and description of microinjection protocols are discussed elsewhere (Hogan et al. 1986).

After this overnight incubation, 15-20 two-cell embryos were introduced into the oviducts of pseudopregnant (C3H X 101)F_1 mice.

transgene by hybridization to the 3' *Gabrb3* probe, 218/219. In *Bam*HI digests, this probe detects a 9.5-kb wild-type genomic fragment and a 1.7-kb fragment in *BAPβ3* transgenics.

Results and Discussion

Transgene Construct Expressing Rat *Gabrb3* cDNA

The transgene construct, *BAPβ3* (**Fig. 2**) is a 6.5-kb *Kpn*I-*Xba*I fragment containing the following elements: a 5' human β-actin promoter along with 5' untranslated sequence and first intervening sequence; a 2-kb rat *Gabrb3* cDNA (Ymer et al., 1989); and an SV40 poly A site. The rat cDNA starts 48 bp upstream of the start codon and has 1490 bp of coding region. This *Gabrb3* cDNA has previously been co-expressed with a $GABA_A$ receptor α subunit gene in *Xenopus* oocytes and was shown to form active $GABA_A$ receptor chloride channels (Ymer et al., 1989). The human β-actin promoter is known to direct high expression of transgenes in a wide variety of tissues (Gunning et al., 1987); the promoter and 5' transcription elements of the rat and human *Gabrb3* genes were recently characterized and were shown to direct strong expression of the endogenous gene (Kirkness et al., 1993).

Recovery of $p^{4THO-II}$ / $p^{4THO-II}$ Mice that Exhibit Normal Palate Development

Neonatal mice homozygous for the $p^{4THO-II}$ deletion have a cleft palate and typically die at birth (Culiat et al., 1993). To test whether a highly expressed *Gabrb3* gene could correct the CP phenotype in $p^{4THO-II}$ homozygotes, *BAPβ3* was microinjected into fertilized eggs obtained from the cross of FVB females (+/+) to $p^{7R75M}/p^{4THO-II}$ males (**Fig. 3**). p^{7R75M} is a radiation-induced viable allele of the *p* locus that produces a phenotype in which eye and coat color are darker in homozygotes than in $p^{7R75M}/p^{4THO-II}$ heterozygotes (Culiat et al., 1993). This allowed rapid determination of genotypes based on intensity of coat and eye color in all offsprings of founders except those of the genotypes $+/p^{7R75M}$ or $+/p^{4THO-II}$, which are indistinguishable from wildtype (+/+).

Pups born from microinjected eggs were screened for the presence of the transgene by hybridization of tail DNAs to the probe 218/219 (Nicholls et al. 1993), which is a 258-bp PCR product from rat genomic DNA corresponding to a 3' region of *Gabrb3* that has the least homology to other β subunit genes (Ymer et al., 1989). This probe detects a 9.5-kb *Bam*HI fragment in wild-type genomic DNA and a 1.7-kb fragment in *BAPβ3* transgenics (**Fig. 2**). One founder, designated as *TgN(Gabrb3)5023Rn*, was identified among 35 pups born from the first microinjection experiment. When crossed to a $p^{7R75M}/p^{4THO-II}$ male, this female was shown to be $+/p^{7R75M}$; *BAPβ3*/+. Transgenic progeny of this testcross that were also intermediate in color (i.e., $p^{7R75M}/p^{4THO-II}$;

BAPβ3/+) were therefore mated to non-transgenic $p^{7R75M}/p^{4THO-II}$ mice to generate mice of the desired $p^{4THO-II}/p^{4THO-II}$; *BAPβ3/+* genotype. Significantly, eight pink-eyed mice with normal palates were recovered from these latter matings (progeny of females #6, #5, #3; refer to **Table 2**). The litter into which two of these rescued mice were born is shown in **Fig. 4**. DNA analyses (**Fig. 5 lanes a-g**) indicated that these mice lacked the endogenous copy of *Gabrb3* and that 7 out of the 8 mice carried the *BAPβ3* transgene (i.e., were $p^{4THO-II}/p^{4THO-II}$; *BAPβ3/+*). Other than being smaller than their littermates (**Fig. 6**), these mice did not display obvious neurological defects nor any readily detectable aberrant phenotypes.

One rescued male ($p^{4THO-II}/p^{4THO-II}$; *BAPβ3/+*) was also mated to a $p^{7R75M}/p^{4THO-II}$; *BAPβ3/+* female, yielding four additional $p^{4THO-II}/p^{4THO-II}$ pups with normal palates (progeny of female #53; refer to **Table 2**). DNA analysis indicated that all four carried the *BAPβ3* transgene (**Fig. 5**).

Further confirmation of rescue of cleft palate by the *BAPβ3* transgene was obtained from a second expressing founder line, *TgN(Gabrb3)5058Rn*, derived from a second microinjection experiment. The *BAPβ3/+* founder female was crossed to a $p^{7R75M}/p^{4THO-II}$ male, to yield a pink-eyed $p^{4THO-II}/p^{4THO-II}$ pup (out of 27 total pups observed) with normal palate, who was also transgenic (thus, the founder of the *5058Rn* line is $+/p^{4THO-II}$; *BAPβ3/+*). The phenotype of this rescued mouse is similar to those recovered from the *5023Rn* line.

To date, a total of 12 $p^{4THO-II}/p^{4THO-II}$ mice with normal palates

have been recovered from all crosses from the *TgN(Gabrb3)5058Rn* line presented in **Table 2**. Eleven of these mice carry only the transgene copy of *Gabrb3,* whereas one carried no *Gabrb3* sequences (**Fig. 5 lane g**). Since the penetrance of the $p^{4THO-II}$ mutation is estimated to be 95%, we expect ~1/80-1/100 escapers in a cross of $p^{4THO-II}$ heterozygotes (Culiat et al., 1993; 1994). We observed a single escaper and 7 rescued mice among a total of 113 progeny from the $p^{7R75M}/p^{4THO-II}$; *BAPβ3*/+ X $p^{7R75M}/p^{4THO-II}$ cross (**Table 2**).

As a control for genetic background decreasing the penetrance of the $p^{4THO-II}$ mutant phenotype, FVB- +/+ females were crossed to $p^{7R75M}/p^{4THO-II}$ males and the $+/p^{4THO-II}$ F_1 progeny were then crossed to $p^{7R75M}/p^{4THO-II}$ mice to recover $p^{4THO-II}$ homozygotes. The $+/p^{4THO-II}$ F_1 progeny were identified by probing *Bam*HI-digested tail DNAs with the 0.8 probe (*D7Cwr15* locus), which detects a restriction fragment length polymorphism between p^{7R75M} and FVB (+). The crosses in this assay for the effect of the FVB background on the penetrance of $p^{4THO-II}$, are identical to those done in the phenotype-rescue experiment, except that the *Gabrb3* transgene is absent. Phenotypes of neonates were recorded, and progeny with unpigmented eyes that showed no milk in their stomachs after birth, were dissected to check for cleft palate. The frequency of "escapers" was calculated and compared to the data for penetrance in the original $p^{4THO-II}$ line (that had no FVB/N background contribution). This assay for the the effect of the FVB background on the penetrance of $p^{4THO-II}$ was conducted by D. K. Johnson and D. Carpenter. The results are summarized in **Table 3**. These control crosses

demonstrated that the FVB/N background has little effect in the penetrance of the *cp1* defect since no $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ "escapers" were recovered from 92 progeny examined to date (**Table 3**). Based on the earlier calculation of the penetrance of $p^{4THO\text{-}II}$, we expect zero or one escapers out of this number of progeny from a cross of $p^{4THO\text{-}II}$ heterozygotes.

Expression of the *Gabrb3* Endogenous Gene and Transgene

In mice, the endogenous 5.5-kb *Gabrb3* transcript is expressed at high levels in brain, but is also expressed at lower levels in tissues such as liver, kidney, and spleen (Kirkness and Fraser, 1993; and **Fig. 7**). An alternative 3.0-kb transcript is also detectable in brain and kidney. **Fig. 7** shows expression of the *BAPβ3* transgene (a 2.4-kb transcript) in the brain, liver, kidney, spleen, and intestine in a $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ transgenic mouse rescued from lethal cleft palate. As expected, there is no endogenous *Gabrb3* transcript expressed in this $p^{4THO\text{-}II}$ homozygote. *BAPβ3* is also expressed in fetal heads at 17.5 days of gestation (data not shown).

It is interesting that the dominant *Gabrb3* transcript in mouse brain is 5.5 kb in length, in contrast to the 2.4-kb message prevalent in rat cerebellum (Ymer et al., 1989). A higher molecular weight RNA (6.0 kb) has also been observed in rat brain (Ymer et al., 1989). Northern-blot analysis of poly-A^+ RNA prepared from mouse palate shelves at E14.5, the

critical stage of palate-shelf elevation and fusion, shows only the characteristic murine 5.5-kb endogenous transcript expressed abundantly enough to be detectable by Northern blot analysis (D. Johnson, unpublished data). It is not known whether smaller transcripts are present, but at undetectable levels, in this tissue or are completely absent. We note, however, that the smaller rat *Gabrb3* message is sufficient for providing the functions necessary for correcting the cleft-palate defect and also for alleviating (or perhaps correcting) the neurological defect displayed by the rare $p^{4THO\text{-}II}$ homozygotes that manage to escape CP.

Expression of the *BAPβ3* transgene in tissues where *Gabrb3* is not normally expressed (e.g., in muscle, data not shown) appears to have no easily recognizable adverse effects. This result is perhaps not surprising, given that other $GABA_A$-receptor subunit genes might not be expressed in these tissues, thereby making inappropriately expressed *Gabrb3* polypeptide functionally inconsequential.

Not all $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ mice that carry the transgene get rescued from lethal cleft palate (**Fig. 8**), possibly because the expression of the transgene is lower or more variable than the endogenous level **(Fig. 7).** Thus this level of expression might be at the threshold needed for normal palate development to occur, and in some fetuses other factors are present that prevents rescue.

Imprinting of the *BAPβ3* Transgene

To get enough data for a better correlation between the presence of the transgene and normal palate in $p^{4THO-II}$ homozygotes (rescued mice) three transgenic $p^{7R75M}/p^{4THO-II}$ males were each mated to $p^{7R75M}/p^{4THO-II}$ females. Each pregnant female was dissected at E17.5 and fetuses were examined for the palate phenotype. DNA was isolated from all pink-eyed fetuses to screen for the presence of the transgene. There was no rescue observed in this experiment, in direct contrast to the experiment where liveborn transgenic pink-eyed mice were generated. Since in the latter, the source of the transgene were all females, it raised the possibility that the transgene was imprinted when passed through the male germline. To test this possibility 24 crosses of $p^{7R75M}/p^{4THO-II}$; *BAPβ3*/+ X $p^{7R75M}/p^{4THO-II}$ were conducted wherein 12 pairs had the female as the transgenic parent and the other 12 had the male as the source of the transgene. Phenotypes of all progeny were recorded and DNA analyses were conducted on all pink-eyed progeny to confirm presence of the transgene. This assay for the imprinting of the *BAPβ3* transgene was conducted by D. Johnson and D. Carpenter. The results are summarized in **Table 4**. In the 149 progeny recovered so far from the crosses where the transgene was inherited from the female, 3 pink-eyed mice with normal palate were present. None was recovered in the 77 progeny born from the crosses where the transgene was inherited from the male. Since the endogenous *Gabrb3* gene is not imprinted in mouse brain (Nicholls et al., 1993), this observation is not reflective of the *in vivo* imprinting status of

the gene. Analysis of RNA from fetal heads at 17.5 days of gestation showed that the transgene was expressed when transmitted from either parent, suggesting that the imprinting could be tissue specific (data not shown). For the purposes of this study, this possibility was not explored further.

Other Phenotypes Associated with Homozygosity for the *p4THO-II* Deletion

Previous studies (Culiat et al., 1993) have indicated that the CP phenotype in *p4THO-II/p4THO-II* homozygotes is not completely penetrant. Unlike the transgenic rescued homozygous animals described above, homozygous escapers (that completely lack *Gabrb3* polypeptides) are runted, nervous, and exhibit pronounced jerky, shaking movements. Such escapers also lack the γ3 and α5 subunits of the $GABA_A$ receptor, because the genes encoding these subunits are also contained within the *p4THO-II* deletion (Phillips, 1973; Nakatsu et al., 1993; Culiat et al., 1994). However, we have shown that deletion of the γ3 and α5 polypeptides has no obvious neurological or physical consequence in mice (Culiat et al., 1994). Because the *BAPβ3* transgene corrects both neonatal cleft palate and the juvenile nervousness in transgenic *p4THO-II* homozygotes, we propose that lack of *Gabrb3* is solely responsible for the secondary juvenile neurological phenotype observed in the rare animals that escape the temporally earlier, primary, neonatal CP defect caused by

Gabrb3 deficiency.

On the other hand, the small size of the rescued animals may be attributed to another gene in the $p^{4THO\text{-}II}$ deletion. This growth-retardation phenotype is unlikely to be due to the effects of the *BAPβ3* transgene because the rare $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ "escapers", which are non-transgenic, display a similar smaller-size phenotype. Moreover, $p^{7R75M}/p^{4THO\text{-}II}$ or p^{7R75M}/p^{7R75M} mice carrying *BAPβ3* transgenes do not exhibit any growth retardation when compared to their non-transgenic littermates. Thus, this growth retardation may be due to another locus that maps between the distal breakpoints of the p^{83FBFo} and $p^{4THO\text{-}II}$ deletions (Culiat et al., 1993).

The location of the gene responsible for this reduction in size of $p^{4THO\text{-}II}$ homozygotes was further narrowed down by making the cross $p^{7R75M}/p^{4THO\text{-}II}$; *BAPβ3*/+ X $p^{226THO\text{-}I}/p^{7R75M}$. A compound heterozygote with the genotype $p^{226THO\text{-}I}/p^{4THO\text{-}II}$; *BAPβ3*/+ was recently recovered. This pink-eyed pup did not have obvious neurological defects and was rescued from cleft palate but still manifested the growth retardation phenotype. Since the distal endpoint of the $p^{226THO\text{-}I}$ deletion lies proximal to *Gabrb3* coding regions, but does affect *Gabrb3* transcription (Culiat et al., 1993), the gene responsible for the growth retardation defect must map within the ~100 kb *Gabra5-Gabrb3* interval (see **Section IV.**). An equally likely alternative would require that this growth retardation stems from a minor upset in normal GABAergic pathways involving the ß3 subunit such that this phenotype would be realized in small $p^{4THO\text{-}II}/p^{4THO\text{-}II}$; *BAPβ3*/+ mice that have been rescued

from the CP and neurological defects. In other words, the growth retardation is a consequence of aberrancies in *Gabrb3* expression that are not fully corrected in the currently available *Gabrb3* transgenic lines.

Neurotransmitters in Secondary Palate Development

Current data support a model wherein a tremendous diversity of $GABA_A$ receptor subtypes is generated by different combinations of 4-5 polypeptide subunits (DeLorey and Olsen, 1992; Olsen and Tobin, 1990; Burt and Kamatchi, 1991). So far, 16 subunit genes have been isolated and classfied into five homology classes: α1-6, β1-3, γ1-3, δ1, ρ1-2 (DeLorey and Olsen, 1992; Olsen and Tobin, 1990; Burt and Kamatchi, 1991). Members of the same class exhibit 70-80% homology of amino acid sequence as compared to 30-40% between different classes. The presence of alternative transcripts of particular subunit genes contributes another level of diversity (DeLorey and Olsen, 1992; Cutting et al., 1992; Whiting et al., 1990). Subunit genes are differentially expressed depending on developmental stage and type of tissues (Laurie et al., 1992). Although they are primarily distributed in component tissues of the central nervous system, data are accumulating that reveal the expression of these genes in other tissues (Kirkness and Fraser, 1993; DeLorey and Olsen 1992), thereby supporting the idea that $GABA_A$ receptors (or at least subsets of the receptor polypeptides) may also mediate non-neuronal processes. The heterogeneity in composition and the ability of the

$GABA_A$ receptor to interact with a wide array of ligands (DeLorey and Olsen, 1992; Olsen and Tobin, 1990; Burt and Kamatchi, 1991) hint at a great functional versatility.

It is not clear at this point whether the binding of GABA itself to a $GABA_A$ receptor containing the β3 subunit is the necessary event in palate development. It is formally possible that another ligand necessary for palatogenesis competes with GABA for the same receptor subtype or subunit polypeptide. However, earlier data showing the specific effects of different neurotransmitters in palate development seem to argue that GABA itself is involved. Other neurotransmitters, such serotonin and acetylcholine, promote palate-shelf elevation *in vitro*, whereas dopamine and norepinephrine have no effect (Wee and Zimmerman, 1983; Zimmerman and Wee, 1984; Zimmerman, 1985). Moreover, serotonin receptors are expressed and active in epithelia of developing mouse palate, oral cavity, and face (Lauder and Zimmerman, 1988). Because our data indicate a role for a $GABA_A$ receptor in facial development, it will be interesting to ascertain whether disruption of the genes encoding receptors of other neurotransmitters also cause abnormalities in facial development. Such a broadened role for neurotransmitters in embryonic and fetal development would be consistent with the striking observation that a majority of teratogens have neuropharmacologic activity that either alters neurotransmitter levels or competes/interferes with neurotransmitter/receptor interactions (e.g. sedatives, anticonvulsants, alcohol, tranquilizers) (Wee and Zimmerman, 1983; Zimmerman and Wee, 1984; Zimmerman, 1985). This broadened role would also be consistent

with studies in a wide variety of organisms that suggest that neurotransmitters serve as growth regulators and promoters of differentiation (Lauder, 1993). An excellent demonstration of this phenomenon is a recent study showing that in pre-pubertal monkeys, GABA inhibits the release of luteinizing hormone-releasing hormone (LHRH), the key factor for the onset of puberty in primates (Mitsushima et al., 1994). This inhibitory effect is mediated by the binding of the neurotransmitter to the $GABA_A$ receptor because addition of specific blocking agents to the receptor relieves the inhibition resulting in LHRH release.

Mouse-Human Homologies

The chromosomal region surrounding *Gabrb3* in mouse chromosome 7 is homologous to human chromosome 15q11-q13, which is associated with Prader-Willi (PWS) and Angelman (AS) syndromes as well as with a number of other phenotypes (Nicholls et al., 1993; Wagstaff et al. 1991, Brilliant, 1992). We have previously suggested that, because of the mapping of *cp1* to this highly conserved linkage group, 15q11-q13 loci should be considered when evaluating human families/populations for inherited craniofacial abnormalities (Culiat et al., 1993). We also have presented data supporting the idea that defects in *Gabrb3* are unlikely to be the cause of AS (Culiat et al., 1993; 1994). There is a remarkable conservation of coding regions (94-97%) and 5' regulatory regions (80-

90%) between the human and rat *Gabrb3* genes (Kirkness and Fraser, 1993), which likely reflects similarity in expression and function. Although previous studies in humans have not been conclusive, maternal intake of the GABA agonist diazepam has been associated with oral clefting (Saxen and Saxen, 1975; Safra and Oakley, 1975). Although it is known that facial maldevelopment is not a major clinical manifestation of PWS or AS (Butler, 1990; Clayton-Smith and Pembrey, 1992), nonetheless we suggest that patients with 15q11-q13 abnormalities, especially those heterozygous for a deletion of the *GABRB3* gene, be evaluated for either obvious or subtle defects in facial development and compared with the facial phenotype of patients who do not carry detectable *GABRB3* deletions. Although the β3-related cleft-palate defect is inherited as a recessive with ~95% penetrance in mice, one cannot assume *a priori* that complete loss of gene activity is necessary to observe a similar phenotype in humans. For example, hemizygosity for the *P(D15S12)* gene, the human homologue of the pink-eyed dilution gene in mice, probably causes the hypopigmentation observed in PWS or AS patients who carry long deletions that include the distally mapping *P(D15S12)* gene (Rinchik et al., 1993). Hemizygosity for the *GABRB3* gene in humans could conceivably specify a variably expressed craniofacial and/or neurological phenotype in patients carrying deletions of human 15q11-q13. Furthermore, intragenic *GABRB3* mutations (therefore not including the PWS and AS critical regions) might be found to contribute to familial or sporadic craniofacial abnormalities in human populations.

SECTION V.

TABLES

TABLE 1. Summary of Data on Founder Lines Transgenic for *BAPβ3*

Founder Line [a]	*p* Region Genotype of Founder	Expression of *BAPβ3* Transgene [b]	Frequency of Transmission [c]
TgN(*Gabrb3*)5023Rn (1TGβ3)	$+/p^{7R75M}$	+	66 / 142
TgN(*Gabrb3*)5047Rn (2TGβ3)	$+/p^{7R75M}$	—	3 / 25
TgN(*Gabrb3*)5058Rn (3TGβ3)	$+/p^{4THO-II}$	+	3 / 16
TgN(*Gabrb3*)5071Rn (4TGβ3)	$+/p^{7R75M}$	+	1 / 25
TgN(*Gabrb3*)5074Rn (5TGβ3)	$+/p^{7R75M}$	—	3 / 24
TgN(*Gabrb3*)5115Rn (6TGβ3)	$+/p^{4THO-II}$	[d]	0 / 24

[a] Official name; In parentheses is the abbreviated name used in breeding records.

[b] Northern analyses with a probe for *Gabrb3*, probe 218/219; — designates no expression while + represents presence of transcript from *BAPβ3* transgene.

[c] ratio of transgenic progeny to total number of progeny screened in the cross Founder X $p^{7R75M}/p^{4THO-II}$, where the founder can be either $+/p^{7R75M}$ or $+/p^{4THO-II}$.

[d] Has not been tested because founder does not transmit the transgene.

TABLE 2. Recovery of $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ Mice Rescued from Cleft Palate

Transgenic female parent [a]	$p^{4THO\text{-}II}$ / $p^{4THO\text{-}II}$		Total number of progeny screened [d]
p^{7R75M} / $p^{4THO\text{-}II}$; *BAPβ3* /+	NP [b]	CP [c]	
1TGβ3 #6	1 tg 1 ntg	2 tg 1 ntg	38
1TGβ3 #5	4 tg	—	43
1TGβ3 #3	2 tg	3	32
1TGβ3 #53	4 tg	3	24

[a] First three females (#6, #5, and #3) were mated to p^{7R75M} / $p^{4THO\text{-}II}$; #53 was mated to a rescued male 1TGβ3 #66 ($p^{4THO\text{-}II}$ / $p^{4THO\text{-}II}$; *BAPβ3* /+).

[b] tg (transgenic) or ntg (non-transgenic) $p^{4THO\text{-}II}$ homozygote with normal palate (NP)

[c] Neonates with cleft palate (CP); Very few are recovered because they are cannibalized by mother; Where it is not indicated whether tg or ntg, DNA analysis could not be done because fetus was dead for some time.

[d] Tail DNAs were analyzed by Southern blotting and hybridization to the *Gabrb3* probe 218/219.

TABLE 3. Assaying the Effect of FVB Background on the Penetrance of the *p4THO-II* Mutant Phenotype [a]

	p4THO-II / p4THO-II [b]		Total number of progeny observed
	NP	CP	
observed [b]	0	8	92
expected [c]	0-1	23 [d]	—

a Ongoing experiment by D. K. Johnson;

b Pink-eyed progeny recovered from crosses of $+/p^{4THO-II}$ X p^{7R75M} / $p^{4THO-II}$ where the former are F1 progeny from a cross of FVB– +/+ X p^{7R75M} / $p^{4THO-II}$ mice; Tail DNAs are still being analyzed by southern blotting and hybridization to the *Gabrb3* probe 218/219.

c Prior calculation of the penetrance of the *p4THO-II* mutation predicts one *p4THO-II* homozygote with normal palate (NP) is expected out of 100 progeny of a cross of *p4THO-II* heterozygotes.

d In an intercross of *p4THO-II* heterozygotes, 1/4 of the total progeny (92/4 = 23) will be homozygotes and will exhibit cleft palate (CP). Very few are actually recovered because they are cannibalized by the mother.

TABLE 4. Tabulation of Data Assaying for Imprinting of the *BAPβ3* Transgene [a]

Transgenic parent	*p4THO-II / p4THO-II* [d]		Total number of progeny observed
p7R75M / p4THO-II; BAPβ3 /+	NP	CP	
12 females [b]	3	5	149
6 males [c]	0	8	77

a Ongoing experiment by D. K. Johnson

b each mated to a *p7R75M / p4THO-II* male

c each mated to two *p7R75M / p4THO-II* females

d Pink-eyed progeny recovered from matings, exhibiting either cleft palate (CP) or normal palate (NP). Tail DNAs are still being analyzed by Southern blotting and hybridization to the *Gabrb3* probe 218/219 to check for the presence of the transgene.

SECTION V.

FIGURES

FIGURE 1. Cloning Strategy for Constructing Transgene *BAPβ3*

The *pBAPβ3* plasmid was derived from three different plasmids: *pβ3* (contains the *Gabrb3* cDNA); *pBSSK*; and *pBAP.2* (contains the human β-actin expression cassette). An *Mlu*I site was first added into the multiple cloning site of *pBAP.2*. Then the 4.5-kb *ClaI* expression cassette, which contains human actin promoter-5' regulatory sequences --an intervening sequence-- SV40 poly A site, was excised and inserted into the *Cla*I site of *pBSSK*. These manipulations were necessary because all the original cloning enzyme sites of *pBAP.2* along with *Cla*I cut the *Gabrb3* cDNA. *Gabrb3* cDNA was inserted into the *pBAPBS Mlu*I cloning site via *Mlu*I linkers and the final trangene construct was cleaved from the *pBAPβ3* plasmid by a *Kpn*I-*Xba*I double digest. These enzyme sites of *pBSSK* flank the original *Cla*I site of the cassette and do not cleave the cDNA. The latter was removed from *pβ3* by *Nru*I-*Xba*I digest then the ends were filled in and *Mlu*I linkers were attached. This was then digested with *Mlu*I, and then ligated to *Mlu*I-digested, dephosphorylated *pBAPBS*. The *6.5*-kb *Kpn*I-*Xba*I fragment was excised from a purified plasmid preparation of *pBAPβ3* (restriction map of this *Kpn*I-*Xba*I fragment is illustrated in the next figure). The transgene was separated from vector sequences by electrophoresis in a low melting gel and extraction of the DNA from the agar slice by melting the agar with sodium iodide solution, followed by binding the DNA to glass powder beads. The DNA was then eluted from the beads in 10 mM Tris, 1 mM EDTA.

Restriction enzymes are designated as: *Bam*HI (B), *Cla*I (C), *Kpn*I

(K), *Mlu*I (M), *Nru*I (N) *Xba*I (X). Enzyme sites with asterisks (*) were destroyed by filling-in reactions. BAP represents the human β-actin promoter, 5' regulatory region and intervening sequence, while pA represents the SV40 poly A site.

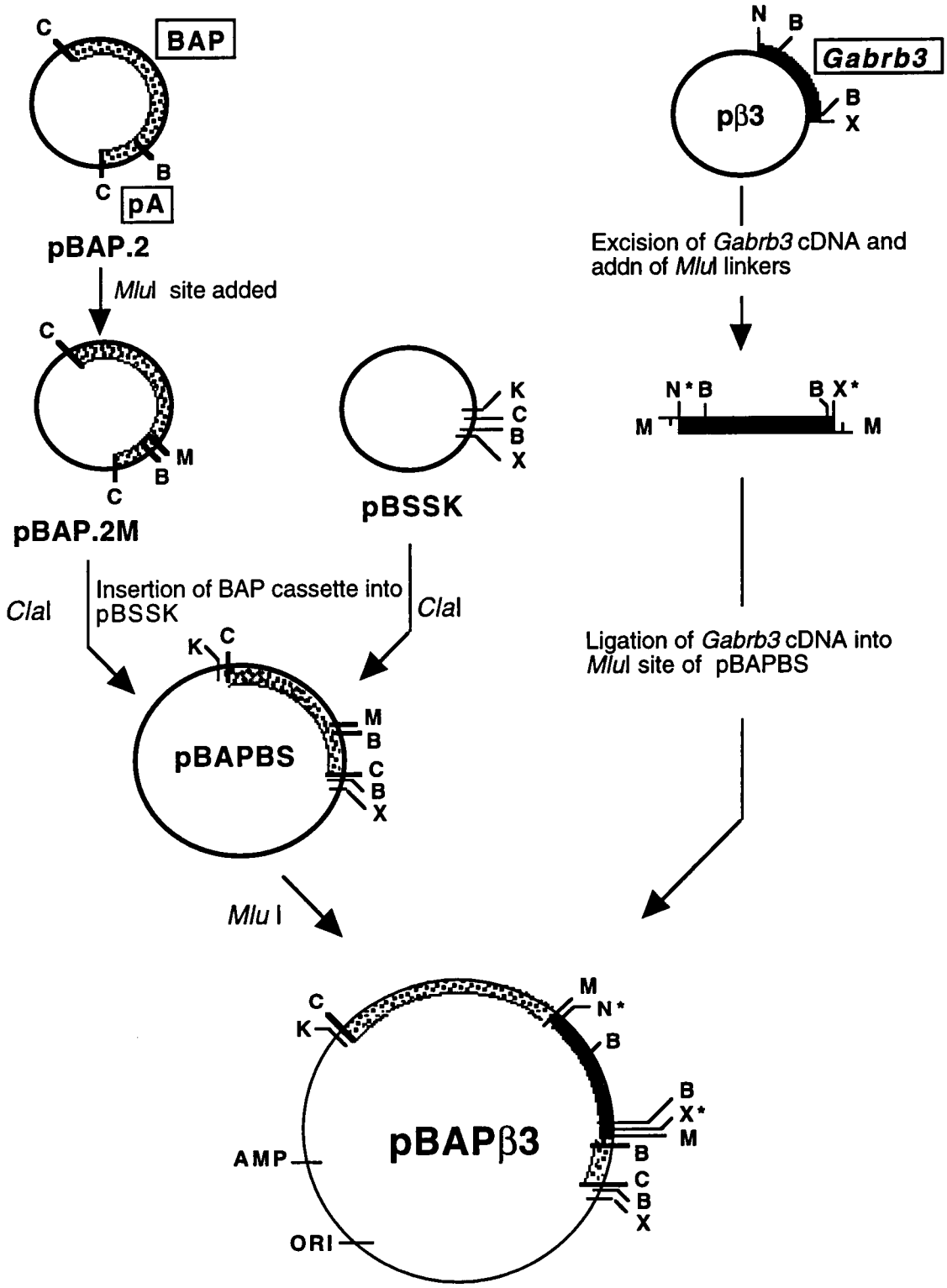

C
BAP
B
C
pA
pBAP.2
MluI site added
C
M
B
C
pBAP.2M
pBSSK
K
C
B
X
ClaI
Insertion of BAP cassette into pBSSK
ClaI
K
C
M
B
C
B
X
pBAPBS
Mlu I
N
B
Gabrb3
pβ3
B
X
Excision of Gabrb3 cDNA and addn of MluI linkers
N*B
B X*
M
M
Ligation of Gabrb3 cDNA into MluI site of pBAPBS
C
K
M
N*
B
B
X*
M
B
C
B
X
AMP
ORI
pBAPβ3

FIGURE 2. Restriction Map of the *BAPβ3* Transgene

Restriction map and components of the *BAPβ3* transgene. The enzyme sites are designated as: *Bam*HI (B), *Cla*I (C), *Kpn*I (K), *Mlu*I (M), *Nru*I (N), and *Xba*I (X). Sites with asterisks (*) were destroyed by filling-in reactions. The regulatory regions controlling the expression of the ~2-kb *Gabrb3* cDNA in this construct include the promoter, 5' untranslated region (5' UTR), and intervening-sequence-1 (IVS-1) of the human β-actin gene as well as the SV40 polyadenylation signal (pA). Presence of the transgene was detected by Southern blotting of *Bam*HI digests of mouse tail DNA, followed by hybridization with a *Gabrb3*-specific probe (218/219). The endogenous 9.5-kb *Gabrb3 Bam*HI fragment is easily differentiated from the 1.7-kb fragment derived from the *BAPβ3* construct.

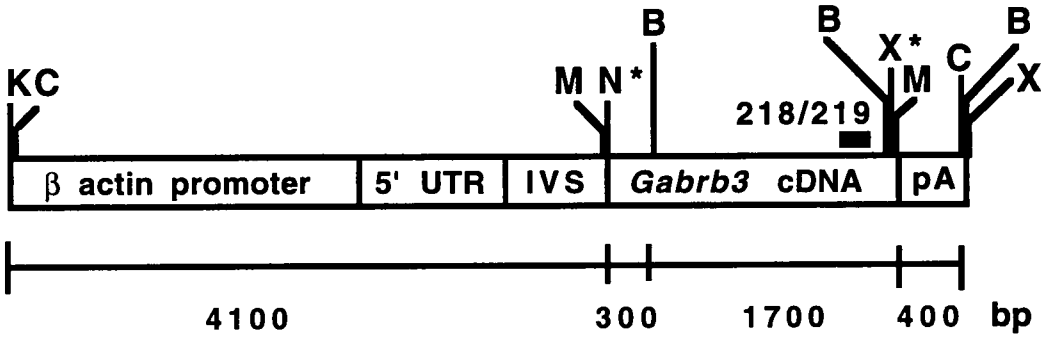

KC
M N*
B
B
X*
M
C
B
X
218/219
β actin promoter
5' UTR
IVS
Gabrb3 cDNA
pA
4100
300
1700
400
bp

FIGURE 3. Genetic Scheme to Recover Transgenic *p4THO-II/p4THO-II* Mice Rescued from Lethal Cleft Palate.

The male pronuclei of fertilized eggs from the cross of FVB– +/+ females X *p7R75M/p4THO-II* males were microinjected with *BAP*β3, the transgene expressing *Gabrb3* under the control of the actin promoter. After division into the two-cell stage, the embryos were introduced into the oviducts of surrogate mothers. The mice that were born were screened for the presence of the transgene by hybridization of the *Gabrb3* probe, 218/219, to Southern blots of tail DNA. Genotypes of mice containing the transgene ("founders") were determined by mating to *p7R75M/p4THO-II* mice. [*p7R75M* is later designated in the diagram as *p*;* presence of transgene is designated as tg.] Note that if founders have the genotype +/*p4THO-II* ; *BAP*β3/+, this cross already produces the pink-eyed *p4THO-II/p4THO-II* ; *BAP*β3/+ mice. On the other hand, a founder with +/*p7R75M* genotype (recognized by producing *p7R75M /p7R75M* progeny detectable by distinct eye and coat pigmentation) will also yield *p7R75M /p4THO-II* ; *BAP*β3/+ mice. These can then be mated to *p7R75M /p4THO-II* mice to generate the *p4THO-II/p4THO-II*; *BAP*β3/+ mice that might be rescued from lethal cleft palate.

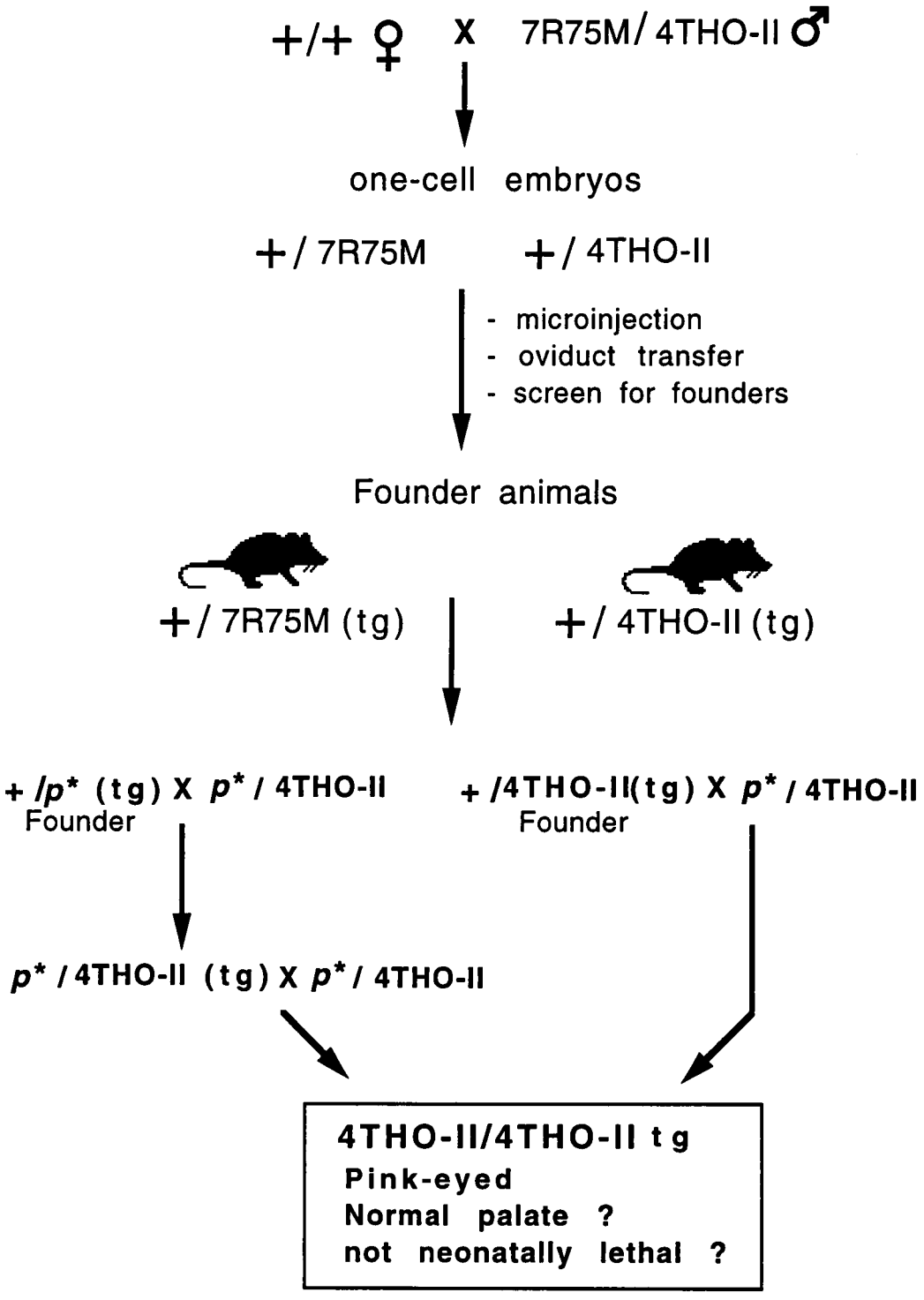

+/+ ♀ X 7R75M/ 4THO-II ♂
one-cell embryos
+/ 7R75M
+/ 4THO-II
- microinjection
- oviduct transfer
- screen for founders
Founder animals
+/ 7R75M (tg)
+/ 4THO-II (tg)
+ /p* (tg) X p* / 4THO-II
Founder
+ /4THO-II(tg) X p* / 4THO-II
Founder
p* / 4THO-II (tg) X p* / 4THO-II
4THO-II/4THO-II tg
Pink-eyed
Normal palate ?
not neonatally lethal ?

FIGURE 4. Litter Showing Two *p4THO-II / p4THO-II*; *BAPβ3/+* Mice Rescued from Neonatally Lethal Cleft Palate.

A litter from the cross *p7R75M/p4THO-II*; *BAPβ3/+* X *p7R75M/p4THO-II*; +/+. The two pink-eyed dilute mice (upper right corner; see arrows) are *p4THO-II / p4THO-II* animals that carry the *BAPβ3* transgene and are rescued from the neonatally lethal cleft-palate defect. The four mice with intermediate coat color (middle) are *p7R75M / p4THO-II* and the lone dark-colored mouse (lower left corner) is *p7R75M/p7R75M* .

FIGURE 5. Southern Blot Analysis of Rescued *p4THO-II / p4THO-II BAPβ3/+* Mice

Southern blot analysis of 10 μg of *Bam*HI-digested DNA from the indicated genotypes: "+/p^{7R75M} Founder" represents the +/p^{7R75M}; *BAPβ3/+* transgenic founder in the 5023Rn line. **Lanes a-k** represent 11 $p^{4THO-II}/p^{4THO-II}$ mice with normal palates (NP) recovered from the genetic strategy described to generate these mice (**Fig. 2, Table 2**). DNAs from the two rescued mice shown in **Fig. 4** are loaded in lanes b and c. Hybridization with a *Gabrb3* probe (218/219) detects the presence of a wild-type 9.5-kb fragment in the control DNAs but not in the $p^{4THO-II}$ homozygotes (lanes a-k). Note that except for one mouse (lane g) all homozygotes with normal palate are transgenic, as indicated by the presence of the 1.7-kb *Bam*HI fragment from the *BAPβ3* construct. Another $p^{4THO-II}$ homozygote with normal palate was recovered (not shown in this blot) and was determined to be transgenic. Eleven out of the 12 $p^{4THO-II}/p^{4THO-II}$ mice that have survived with normal palate (beyond 24 hrs) carry the transgene, supporting the idea that *Gabrb3* is *cp1*.

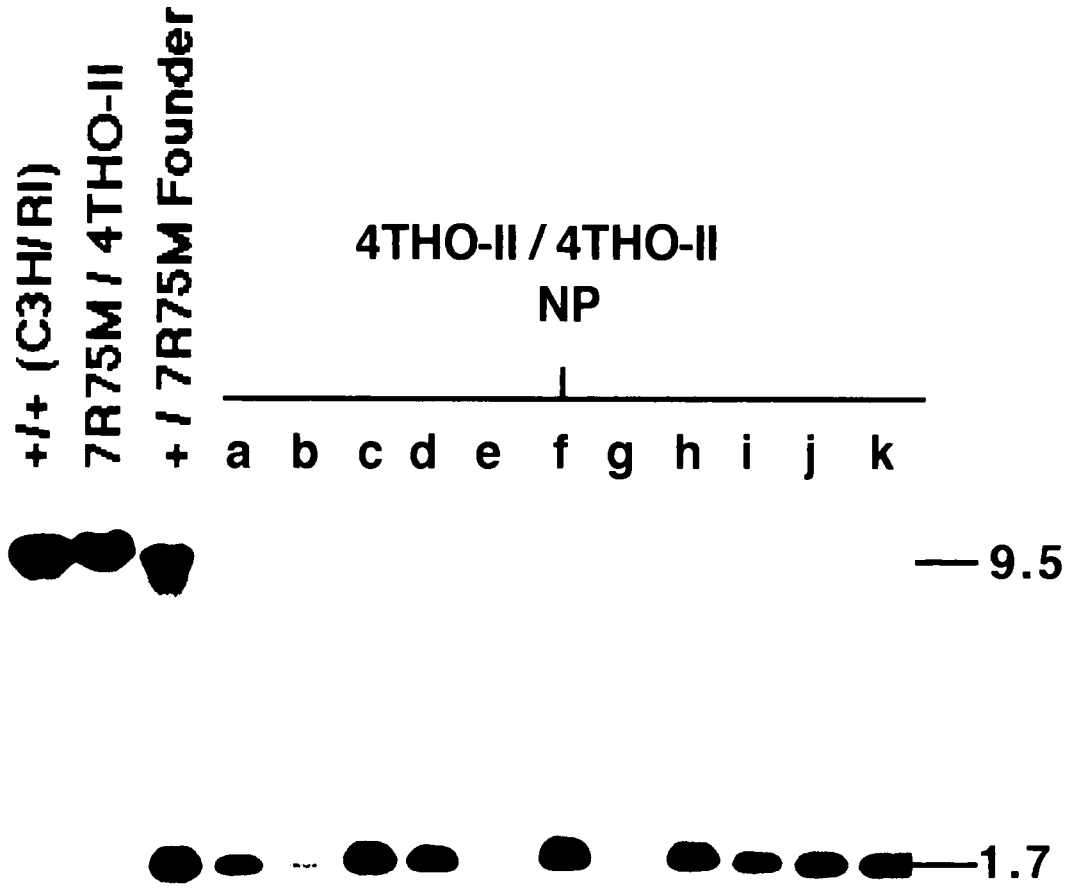

+/+ (C3H/RI)
7R75M / 4THO-II
+ / 7R75M Founder
4THO-II / 4THO-II
NP
a b c d e f g h i j k
9.5
1.7

FIGURE 6. *$p^{4THO-II}$ / $p^{4THO-II}$* Mice Rescued from CP are Smaller than Littermates.

A two-week-old litter from the cross *p^{7R75M} / $p^{4THO-II}$; BAPβ3/+* X *$p^{7R75M}/p^{4THO-II}$* ; +/+. The two pink-eyed dilute mice (see arrows) are *$p^{4THO-II}$ / $p^{4THO-II}$* animals that carry the *BAPβ3* transgene and are rescued from the neonatally lethal cleft-palate defect. The four mice with intermediate coat color (middle) are *p^{7R75M} / $p^{4THO-II}$* and the lone dark-colored mouse (lower left corner) is *p^{7R75M}/p^{7R75M}* . Note that the rescued mice are smaller in size compared to the littermates. This growth retardation is associated with the *$p^{4THO-II}$ / $p^{4THO-II}$* genotype rather than the presence of the transgene because transgenic mice of the other genotypes do not display this size defect.

FIGURE 7. Northern Analysis Comparing Expression of *Gabrb3* in Wildtype and Trangenic Mice.

Poly A^+ analysis showing *Gabrb3* expression in various tissues of an adult (C3H/Rl X 101/Rl)F_1 wild-type adult mouse and a two-week-old *p4THO-II/p4THO-II*; *BAPβ3*/+ mouse rescued from CP. Hybridization of *Gabrb3* probe 218/219 detects an endogenous 5.5-kb transcript and a 2.4-kb transcript from the *BAPβ3* transgene. A 3.0-kb alternative *Gabrb3* transcript can be observed in wild-type brain and and kidney RNA. A comparison of the level of *Gabrb3* expression between wild-type and mice rescued from cleft palate, shows that the transgene is expressed at a lower level in brain but higher in liver. In the other tissues tested in this Northern Blot, the differences are not very dramatic. (Lower panel) To compare RNA loaded in each lane, the blot was hybridized to a probe for chick tubulin, which detects a 1.8-kb transcript.

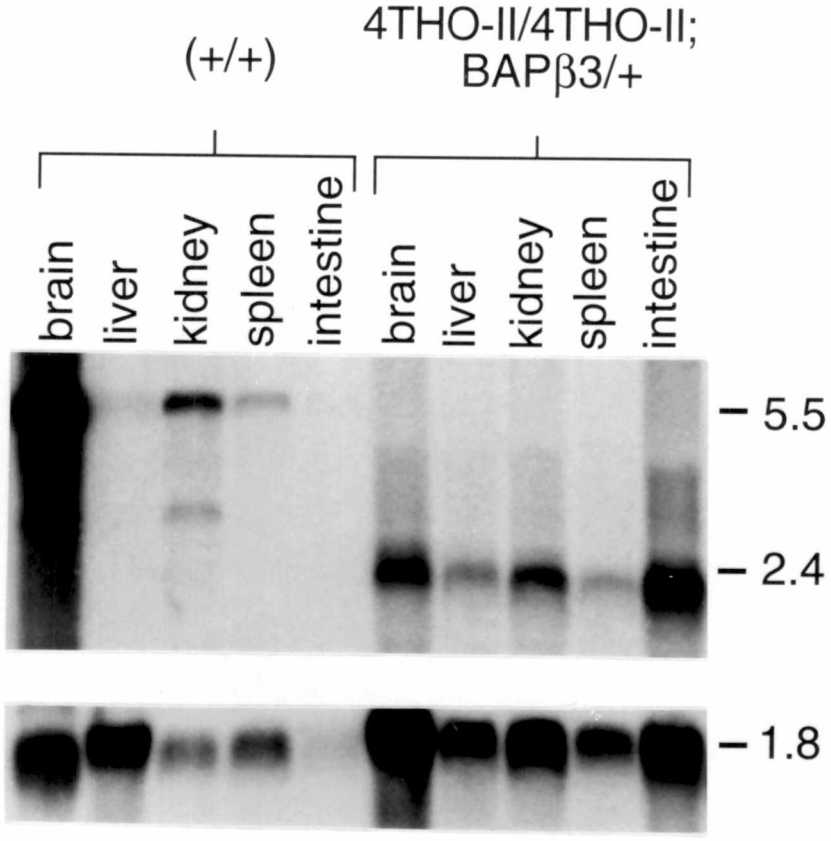
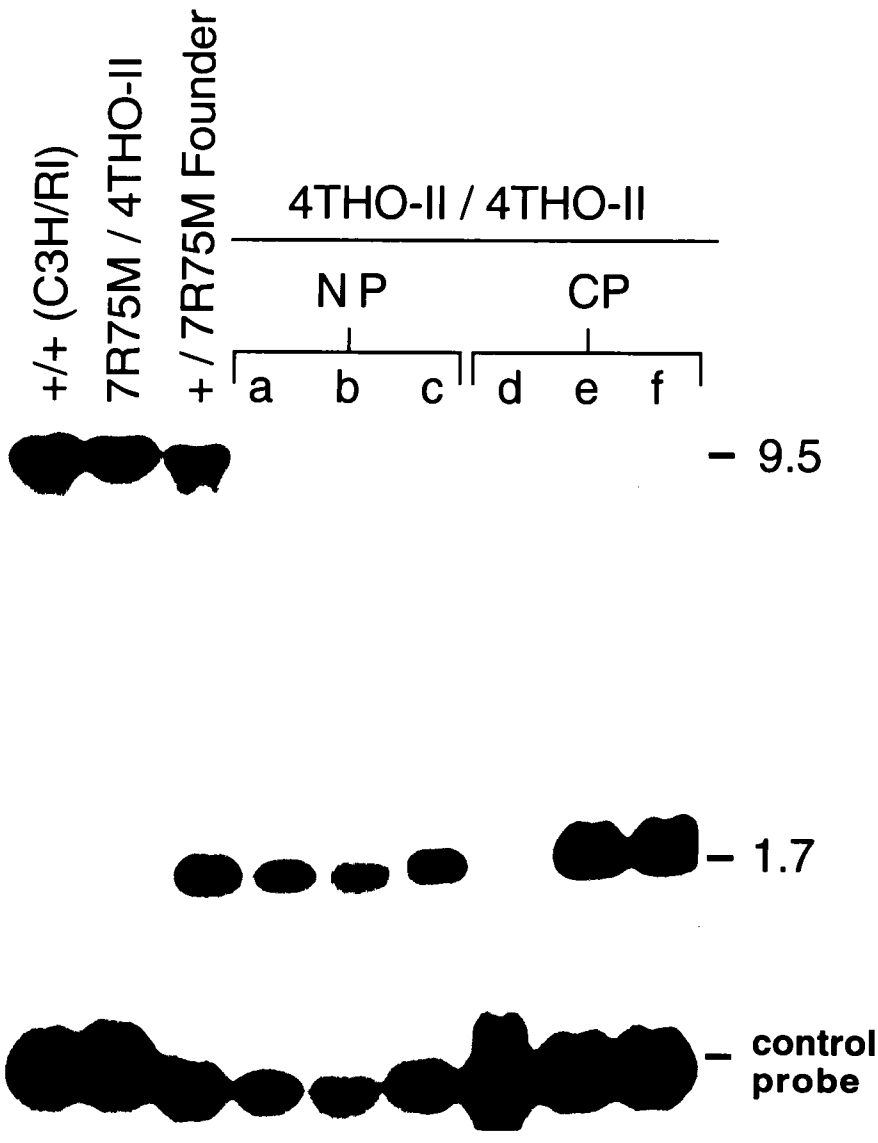

(+/+)
4THO-II/4THO-II;
BAPβ3/+
brain
liver
kidney
spleen
intestine
brain
liver
kidney
spleen
intestine
5.5
2.4
1.8

Figure 8. Southern Blot Analysis Indicating that Not all Transgenic *p4THO-II* / *p4THO-II* Mice are Rescued from CP

Southern blot analysis of 10 μg of *Bam*HI-digested DNA from the indicated genotypes: "+/p^{7R75M} Founder" represents the +/p^{7R75M}; *BAPβ3*/+ transgenic founder. Lanes **a-c** represents three-week-old $p^{4THO\text{-}II}/p^{4THO\text{-}II}$; *BAPβ3*/+ mice with normal palate (NP) while lanes d-f represent three $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ neonates with cleft palate (CP). DNAs in lanes b and c were derived from the two rescued mice shown in **Fig 4.** Hybridization with a *Gabrb3* probe (218/219) detects the presence of a wild-type 9.5-kb fragment in the control DNAs but not in the $p^{4THO\text{-}II}$ homozygotes (lanes **a-f**). Note that all homozygotes with normal palate are transgenic, as indicated by the presence of the 1.7-kb *Bam*HI fragment from the *BAPβ3* construct. Lane **d** has DNA from a neonate with cleft palate and contains no copy of *Gabrb3* gene. Lanes **e** and **f** represent mice that do not get rescued despite the presence of the transgene. Incomplete rescue by the transgene may be due to a threshold effect. The degree of expression of the transgene may just be right at the threshold level needed for palatogenesis to proceed and in some $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ mice other factors may come to play that renders the expression insufficient for normal development of the palate. This blot was hybridized with a control probe, 23.3, that detects a 4.0 *Bam*HI fragment near the *c* lous in chromosome 7 (lower panel).


+/+ (C3H/RI)
7R75M / 4THO-II
+ / 7R75M Founder
4THO-II / 4THO-II
NP
CP
a
b
c
d
e
f
– 9.5
– 1.7
– control probe

VI. DISCUSSION: MOLECULAR MODELS FOR THE INVOLVEMENT OF A $GABA_A$ RECEPTOR IN PALATE DEVELOPMENT

The rescue of *p4THO-II/p4THO-II* mutants from lethal cleft palate by the introduction of a transgene expressing the *Gabrb3* gene confirms our earlier hypothesis that *Gabrb3* is identical to *cp1*, a gene controlling palate development (Culiat et al., 1993). This demonstration of the importance of a specific $GABA_A$ receptor subunit in palatogenesis further led us to propose that there is a particular $GABA_A$ receptor subtype, which includes the β3 subunit, that plays a role in murine palate development (Culiat et al., 1993, 1994). Since a GABA receptor is composed of 4-5 polypeptides, having at least 1 α, 1β, and 1 γ polypeptide subunit (DeLorey and Olsen, 1992; Burt and Kamatchi, 1991; Olsen and Tobin, 1990), it is still necessary to demonstrate formally the existence of the receptor in the relevant tissues of palatogenesis and to deduce what other subunits comprise this specific receptor combination. It is, however, reasonable at this point to presume that the β3 subunit is part of a GABA receptor active in palate development since there are no available data about this subunit being part of some other (non-$GABA_A$) receptor complex. Moreover, the presence of GABA in palate mesenchyme has been demonstrated, indicating that a GABA receptor may indeed be active in this tissue (Wee and Zimmerman, 1985, 1986). This section will present some speculations on how such a receptor can mediate palatogenesis.

With existing knowledge, it is premature to propose a fully detailed model, nonetheless, one can propose some models, and there are key experiments that still need to be done to understand the biological role of *Gabrb3* and to evaluate which models are feasible and testable. Some of these experiments will be discussed at the end of this section.

Considerations for Models on the Role of a $GABA_A$ Receptor in Palate Development

Any model proposed to explain activity of a $GABA_A$ receptor in palatogenesis must account for the earlier teratological and biochemical studies of palate development, and must also incorporate the recent molecular and genetic data. These concerns are summarized below.

(1) Addition of the neurotransmitter GABA or its agonists (eg. diazepam) induces cleft palate in mouse (Miller and Becker, 1975; Wee and Zimmerman, 1983; Katz, 1988).

(2) GABA and diazepam are inhibitory to palate-shelf reorientation; hence, its level in palate mesenchyme specifically decreases during this critical stage (E14.5) in palatogenesis (Wee, Norman and Zimmerman, 1986)

(3) Antagonists (eg., picrotoxin) of the $GABA_A$ receptor reverses the inhibitory/ teratogenic effect of GABA in *in vitro* organ cultures (Wee and Zimmerman, 1983).

(4) Stimulatory neurotransmitters such as serotonin and acetylcholine have the opposite effect of GABA, namely stimulation of the process of palate-shelf reorientation (Zimmerman, 1985; Zimmerman and Wee, 1984).

5) Disruption of the *Gabrb3* gene, presumably interfering with a specific receptor subtype, induces CP (this work).

(6) The $GABA_A$ receptor binds a wide diversity of substances (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; DeLorey and Olsen, 1992).

Model A: A Decrease in GABAergic Activity Signals A Specific Event Necessary for Palate Development

One model that can explain the role of a $GABA_A$ receptor in palate development is that GABA inhibits a specific step in palate-shelf reorientation (eg., keeping another factor from being released or activated). In normal development, GABAergic activity (binding of GABA to the receptor and opening of the integral Cl^- channel) probably decreases during this critical period in palatogenesis in order to allow this specific event to occur. The decrease of GABA prior to palate-shelf reorientation (Zimmerman, 1985) is consistent with this model. Addition of GABA or other substances that activate the GABAergic pathways (eg. agonists like diazepam) would therefore induce cleft palate due to a delay in palate-shelf reorientation. Removal of the receptor is also able to cause cleft

palate perhaps because the decrease in GABAergic activity could be the result of a feedback mechanism mediated by the receptor.

This type of mechanism is plausible since it has been demonstrated recently that GABA inhibits the release of luteinizing-hormone-releasing-hormone (LHRH) in monkeys (Mitsushima et al., 1993). LHRH is necessary for the release of luteinizing hormone to initiate the onset of puberty. In primates, LHRH is already present in the hypothalamus of prepubertal monkeys, but it is not released before the appropriate developmental time for puberty because of a very strong central inhibition. Mitsushima, Hei and Terasawa (1994) showed that it was GABA exerting this control over the LHRH neurosecretory system, probably by blocking action potentials at the neuroterminals of LHRH neurons. In this system, addition of a $GABA_A$ receptor antagonist quickly leads to the release of LHRH. In normal development therefore, GABA has to decrease for puberty to proceed. Three mechanisms for decreasing GABAergic activity at the onset of puberty were proposed: (1) GABAergic synapses with LHRH neurons become reduced; (2) GABA synthesis by the enzyme GAD (glutamate decarboxylase) is decreased; and (3) neural activity of GABAergic neurons are reduced by some mechanism (Mitsushima, Hei and Terasawa, 1994).

There are various growth factors needed for the growth and differentiation of the developing palate shelves, including epidermal growth factors and transforming growth factor-β (reviewed by Ferguson, 1988; Greene et al., 1991). These factors are implicated in the synthesis of hyaluronic acid by palate mesenchyme cells. The accumulation and

hydration of hyaluronic acid is the intrinsic force in the elevation of the palate shelves to the horizontal position above the tongue. Contractility is also important at this stage in palate development, and neurotransmitters such as serotonin and acetylcholine have been implicated in this aspect of palate morphogenesis (Ferguson, 1980). It is possible then that GABA inhibits the release of one of the growth factors that control the synthesis and hydration of hyaluronic or it could counterbalance the effect of the stimulatory neurotransmitters in determining the contractility of palate mesenchyme.

The major difficulty of this model is explaining how a decrease in GABAergic activity (opening of the $GABA_A$ receptor chloride channel) is related to the release of factors needed for palate-shelf reorientation. An alternative mechanism can be proposed wherein the particular $GABA_A$ receptor involved in palate development functions in an entirely different manner than its role in neuronal tissues. For instance, the receptor can function by clearing out GABA from palate mesenchyme during palate-shelf reorientation. This receptor could be part of a pathway that leads to a decrease in the synthesis of GABA (by inhibition of GAD) or the degradation or conversion of GABA to an inactive form. This decrease in GABA can be mediated via secondary messengers in palate mesenchyme. Since there is a specific decrease in the level of GABA at 14.5 days of gestation, there may be another factor that is expressed in palate mesenchyme, at this time, to enhance the "clearing-out" activity of the receptor. It has been proposed that there is a balance of stimulatory (serotonin and acetylcholine) and inhibitory neurotransmitters (GABA) to

determine the contractility of palate tissues during the stage of palate-shelf reorientation (Ferguson, 1980). The receptor that clears out GABA could be responsible for maintaining the right level of the neurotransmitter during palate development. Based on this modification of Model A, the addition of more GABA would be detrimental to palate development because the level may be too high for the receptors to remove, thereby generating cleft palate. A lack of the receptor would also disrupt normal palate development because the GABA could not be cleared out.

In order to investigate whether the decrease of GABA in palate tissues is due to a decrease in the activity of glutamate decarboxylase (GAD), the enzyme that synthesizes the neurotransmitter, inhibitors of GAD such as 3-mercaptopropionic acid (Katoh et al., 1994) can be added to embryos in culture or injected into pregnant mice prior to palate reorientation (E12) and the GABA concentration is assayed in palate tissues at E13-14 (Wee, Norman and Zimmerman, 1986). If the profile of GABA concentration in palate tissues is regulated via the activity of GAD,, there will be a drastic decrease of the neurotransmitter from E13-E14, in the presence of GAD inhibitors. If it is occuring by some other mechanisms (like degradation or conversion to an inactive form) GAD inhibitors will not have a profound effect in the change of the level of GABA in palate tissues at E13-E14.

MODEL B: GABA Competes with a Ligand for a Binding Site in the $GABA_A$ Receptor formed by the β3 Subunit Polypeptide

A second model that can be proposed is that GABA competes with another ligand for the binding site or domain in the $GABA_A$ receptor that is formed by the β3 subunit. The binding of this ligand would be a prerequisite for palate development. Addition of GABA limits or excludes the other ligand from binding to the receptor, and therefore palate development is perturbed. A deficiency in the receptor due to an absence or aberrant level of the β3 subunit polypeptide (i. e. *p* deletion mutants disrupting *Gabrb3*) would also generate cleft palate. This model is consistent with the ability of the receptor to be bound by a wide diversity of compounds and the fact that the β subunit is the major binding site of GABA in the $GABA_A$ receptor complex (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; DeLorey and Olsen, 1992).

One of the limitations of this model lies in accounting for the fact that agonists that have a different major binding domain than GABA itself generate the same CP phenotype. The binding of benzodiazepines to the GABA receptor is governed primarily by the α subunit, whereas the major binding site of GABA is the β subunit (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; DeLorey and Olsen, 1992). A second limitation of this model is its inability to explain the effect of stimulatory neurotransmitters. Moreover, it is necessary to explain how, based on this model, the binding of the other ligand to the $GABA_A$ receptor would facilitate palate development. It is reasonable to assume that activation of the GABAergic

pathway is not the desired effect of the binding of the unknown ligand because most of the existing data show that enhancement of these reactions by the presence of GABA and its agonists is in fact detrimental to the developing palate. One can argue that if there is indeed another unknown ligand that competes with GABA for receptor binding, it is mediating some other event (not simply activation of the Cl^- channel). A possibility is that its binding to the receptor is somehow linked to one or more of the intracellular secondary mesengers that mediate palatogenesis. Examples of these secondary messenger molecules are: cAMP, cGMP, and Ca^{2+} (Ferguson, 1988).

MODEL C: A Novel Receptor Containing the β3 Subunit Polypeptide is Necessary for Palate Development

A third model can be proposed wherein the β3 subunit polypeptide, coded for by the *Gabrb3* gene, is part of a novel membrane receptor in palate mesenchyme. The binding of a still unknown ligand to this receptor activates secondary messengers in the mesenchyme that in turn activate the enzymes that are responsible for the accumulation and/or hydration of hyaluronic acid. Because the β3 subunit is homologous to other β subunits that are part of $GABA_A$ receptors mediating neuronal inhbition, this novel receptor has an intrinsic affinity to GABA and its agonists. The addition of GABA or its agonists blocks the binding of the ligand needed for palate development, thereby generating cleft palate. A lack of the β3

subunit also generates cleft palate because of a deficiency in this novel receptor.

Proposing a model where the β3 subunit is part of a receptor that has no role in neuronal transmission does not account for the fact that in the rescue experiment we have shown that expressing the *Gabrb3* gene in *p4THO-II* homozygotes rescued both the lethal cleft palate in these mutant mice and the neurological defects of the rare escapers of the clefting defect. The data argues for a $GABA_A$ receptor with a dual function--neuronal transmission and palate development. Based on this third model, it can be suggested that the β3 subunit is a component of a $GABA_A$ receptor that performs the neuronal function and another $GABA_A$ receptor-like complex which facilitates palate development.

Further Characterization of the Role of *Gabrb3* in Palate Development

In order to construct a better model, it is important to know when and where the *Gabrb3* subunit is being expressed during palate development. There are four important periods of mammalian palate development (Sulik and Schoenwolf, 1985; Ferguson, 1988) : 1) Neural crest migration and differentiation into embryonic structures that generate the palates shelves; 2) vertical growth of the shelves along the side of the tongue; 3) elevation and reorientation from vertical to horizontal position above the tongue; 4) movement of palate shelves towards each other and subsequent fusion. One approach that can be applied to identify in which

of these stages the receptor is active is by *in situ* hybridization of labelled *Gabrb3* probes (eg., *Gabrb3* antisense oligonucleotides) to sectioned tissues at these different stages of palatogenesis. Probes for *in situ* hybridization can be labelled using radioisotopes (^{35}S) or non-isotopic labels (eg. digoxygenin and biotin). The strengths and limitations of using such probes to visualize the patterns of expression of a gene in sectioned tissues have been discussed by Rosen and Beddington (1993). A great deal of improvement has been made in *in situ* hybridization techniques. A three-dimensional view of the expression of a gene in the entire mouse embryo is possible using the whole-mount non-isotopic *in situ* hybridization described by Rosen and Beddington (1993). This is particularly useful in studying palate development because of the great deal of massive movement of tissues in the craniofacial region of the developing embryo. Monoclonal antibodies directed towards the β3 subunit polypeptide can also be used as an *in situ* probe.

In situ hybridization has been applied effectively to the determination of the distribution of various $GABA_A$ receptor subunit genes (including β3) in mammalian brain (MacLennan et al., 1991; Laurie, Wisden and Seeburg, 1992). In the same manner, *in situ* hybridization of *Gabrb3* probes to slices of mouse palate shelves from E14.0-E15.0 can be done. E14.0-E15.0 is the time that earlier teratological studies report the neurotransmitter GABA exerts its inhibitory effect in palate development. A comparison of palates from wildtype (+/+), mutant (*$p^{4THO-II}/p^{4THO-II}$*) and rescued (*$p^{4THO-II}/p^{4THO-II}$; BAPβ3/+*) mice can be performed with these techniques.

It is necesary to examine more closely the facial phenotypes of the $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ mutant in comparison to the normal embryos (eg. skeletal analysis; comparison of dissected palates from all possible stages of development) to determine when a difference is detectable. Better characterization of the $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ mutant phenotype will resolve two questions: (1) Is cleft palate in these mutants due to a reorientation problem; and (2) Is there an intrinsic defect in the growth, differentiation or migration of the palate shelves *per se* or is the cleft palate a consequence brought about by defects in other structure of the face (eg. increased width the shelves have to cross to fuse, etc)? The knockout of the genes *Msx 1* (Satokata and Maas, 1994) and *Hoxa-2* (Gendron-Macguire et al., 1993; Riiji et al., 1993) have demonstrated that cleft palate can result from abnormalities in the rest of the developing craniofacial region. In the case of *Hoxa-2* null mutants, the loss of gene function affects the development of skeletal elements from the second branchial arch, which is derived from the neural crest (Gendron-Macguire et al., 1993; Riiji et al., 1993). It was postulated that the CP resulted from mechanical stress due to a caudal skeletal defect. Cleft palate in *Msx1* mutants has been attributed to abnormal epithelial-mesenchymal interactions during bone and tooth development (Satokata and Maas, 1994). Skeletal and histological analyses of the $p^{4THO\text{-}II}$ mutation can be accomplished in a manner similar to that described for the *Hoxa-2 and Msx 1* null mutants (Kessel and Gruss, 1991; Gendron-Maguire et al., 1993; Satokata and Maas, 1994).

There are biochemical approaches to isolate other ligands bound by

particular receptors. The extracellular portion of the β3 subunit gene can be bound to a column and extracts from palate tissues and surrounding structures can be bound to it. Pharmacological studies that identify what specific drugs are able to bind the β3 subunit can also show the potential molecular structure of the ligand that could be necessary for palate development.

Data from further characterization of the biological role of *Gabrb3*, along with those of the recently reported null mutants that display cleft palate (Satokata and Maas, 1994; Proetzel and Doestchman, 1994) will contribute greatly to unraveling some of the complexity of the etiology of facial clefting. The screening of patients with CP for mutations in any of these genes will set a precedent in the application of mouse craniofacial development data to the human population in order to understand, diagnose, and minimize the incidence of this common birth defect.

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GENERAL APPENDIX

TABLES

TABLE 1. Hybridization Probes

Probe	Locus	Size of Fragment [a]	Reference [b]
218/219	*Gabrb3*	nt 223-480; cDNA	Nicholls et al., 1993
280/281	*Gabrb3*	nt 81-570; cDNA	
300/301	*Gabra5*	nt 1194-1377; cDNA	Culiat et al., 1993
385/386	*Gabra5*	nt 207-335; cDNA	
385/387	*Gabra5*	nt 207-537; cDNA	
304/305	*Gabrg3*	nt 1046-1352; cDNA	Culiat et al., 1994
GRV-Right	**c**	*YGO6110* 0.5-kb *Eco* RV fragment	—
GRV-Left		*YGO6110* 0.4-kb *Eco* RV fragment	—
GO713-Right	**c**	0.5-kb *Rsa*I fragment	—
GO713-Left	**c**	2.2-kb *Bam*HI fragment	—
pYAC4-Right	**d**	1.6-kb *Pvu*II-*Bam*HI	Burke et al., 1987
pYAC4-Left	**d**	2.6-kb *Pvu*II-*Bam*HI	Burke et al., 1987

TABLE 1. Continued

Probe	Locus	Size of Fragment[a]	Reference[b]
DN10	*p (D15S12h)*	3.4-kb *Eco*RI	Rinchik et al., 1993
0.8	*D7Cwr15*	0.8-kb	D. Johnson, unpublished
MuDN34	*Znf127 (D15S9h-1)*	1.4-kb *Eco*RI	Nicholls et al., 1993
c23	dilute	nt 1549-3928	Mercer et al., 1991
23.3	*Emv-23*	1.4-kb *Eco*RI-*Hae*III	Rinchik et al., 1989

a Restriction fragment or extent of PCR product based on sequence; nt= nucleotide

b Some probes generated in this study were published (Culiat et al., 1993, 1994) and the rest with no reference given are unpublished

c YAC endclones

d Sequences from pBR322 specific to either left or right arm of the pYAC4 vector

TABLE 2. Description of PCR-Derived Probes for *Gabra5*, *Gabrb3*, and *Gabrg3*

Probe	Locus and region probed	size (bp)	PCR Template [a]	Features	Reference of sequences [b]
218/219	3' *Gabrb3*	258	rat genomic	cytoplasmic domain; least homology to other subunits	Wagstaff et al., 1991
280/281	5' *Gabrb3*	490	mouse cDNA	signal peptide sequence; variable region	Ymer et al., 1989
300/301	3' *Gabra5*	183	rat cDNA	cytoplasmic domain; least homology to other subunits	Krestchatisky et al., 1989
385/386	5' *Gabra5*	128	mouse cDNA	113 nt upstream of start codon; variable region	Krestchatisky et al., 1989
385/387	5' *Gabra5*	330	mouse cDNA	very conserved among γ-subunit genes of $GABA_A$ receptor	Krestchatisky et al., 1989
304/305	3' *Gabrg3*	306	mouse cDNA	cytoplasmic domain; least homology to other subunits	Wilson-Shaw et al., 1991

a cDNA templates were first strands reverse transcribed from total adult brain RNA

b Reference where DNA sequence was obtained for design of PCR primers.

VITA

Cymbeline Tancongco Culiat is the eldest daughter of Crispino and Cinderella Tancongco. She was born on May 19, 1961 in Cagayan de Oro City, Philippines. Supported by scholarships from the Philippine government, she graduated from Philippine Science High School in June of 1978 and later obtained a Bachelor of Science degree in Cell Biology (August, 1981) and a Master of Science degree in Genetics (July 1988) from the University of the Philippines at Los Banos (UPLB). While studying for an M. S. Degree in Genetics at UPLB, she taught basic courses in Genetics and Molecular Biology at the same institution and was promoted to the position of assistant professor in 1990. In the fall of 1991, she started pursuing a Doctor of Philosophy degree at the University of Tennessee Graduate School of Biomedical Sciences at Oak Ridge and completed it in December of 1994. As a Ph. D. student, she had the honor of being awarded membership in the Phi Kappa Phi Honor Society in 1992; the Hilton A. Smith Graduate Fellowship in 1993; and the Chancellor's citation for professional promise in 1994. In the spring of 1995, she will begin working on a research project on mouse neurological mutations, as a postdoctoral fellow, at the Biology Division of Oak Ridge National Laboratory.