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The γ-Aminobutyric Acid Receptor γ3 Subunit Gene (GABRG3) Is Tightly Linked to the α5 Subunit Gene (GABRA5) on Human Chromosome 15q11–q13 and Is Transcribed in the Same Orientation

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$GABA_A$ receptors are heterooligomeric ligand-gated ion channels that mediate the effect of the inhibitory neurotransmitter γ-aminobutyric acid. The $GABA_A$ receptors consist of at least 15 different receptor subunits that can be classified into 5 subfamilies (α, β, γ, δ, ρ) on the basis of sequence similarity. Chromosomal mapping studies have revealed that several of the $GABA_A$ receptor subunit genes appear to be organized as clusters. One such cluster, which consists of the $GABA_A$ receptor β3 (GABRB3) and α5 (GABRA5) subunit genes, is located in chromosome 15q11–q13. It is shown here that the $GABA_A$ receptor γ3 subunit gene (GABRG3) also maps to this region. Lambda and P1 phage clones surrounding both ends of GABRG3 were isolated; the clones derived from the 5′ end of GABRG3 were linked to an existing phage contig spanning the 3′ end of GABRA5. The two genes are located within 35 kb of each other and are transcribed in the same orientation.

INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain, and its effect is mediated primarily through $GABA_A$ receptors. Binding of the ligand to these receptors causes an influx of chloride ions, thus changing the membrane potential of neurons (MacDonald and Olsen, 1994). $GABA_A$ receptors are heterooligomeric structures consisting of several different subunits and appear to be functionally and structurally heterogeneous. Several genes for human, bovine, and rodent receptor subunits have been cloned (Tyndale *et al.*, 1994). By sequence comparison these units have been grouped in several classes, containing several distinct members (α1–6, β1–3, δ, γ1–3, ρ1–2). Spatial variation in the expression pattern of these different subunit types and subtype genes may account for the pharmacological and biochemical diversity of $GABA_A$ receptors (reviewed in Olsen and Tobin, 1990; Cutting *et al.*, 1991).

Several subunit genes have been mapped, and there is evidence that they are organized as clusters on different chromosomes: the genes for subunits α2, β1, and γ1 have been assigned to human chromosome 4p12–p13; α1, α6, β2, and γ2 to human chromosome 5q34–q35; ρ1 and ρ2 to human chromosome 6q14–q21; and α5 and β3 to human chromosome 15q11–q13 (Buckle *et al.*, 1989; Dean *et al.*, 1991; Kirkness *et al.*, 1991; Wagstaff *et al.*, 1991a; Wilcox *et al.*, 1992; Cutting *et al.*, 1992; Knoll *et al.*, 1993; Hicks *et al.*, 1994; Russek and Farb, 1994).

The chromosomal region 15q11–q13 is frequently deleted in patients with either Prader–Willi syndrome (PWS) or Angelman syndrome (AS). The deletions in patients with these two clinically distinct genetic disorders cannot be distinguished by cytogenetic methods (Ledbetter *et al.*, 1981; Magenis *et al.*, 1987), but they differ in the parental origin of the involved chromosome. In PWS, deletions are found exclusively on the paternal chromosome (Butler and Palmer, 1983) and in AS exclusively on the maternal chromosome (Knoll *et al.*, 1989). These observations indicate that the PWS/AS chromosomal region is subject to parental imprinting, containing genes that are differentially expressed from the maternal and paternal alleles. The genes of the $GABA_A$ receptor subunits α5 and β3

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(GABRA5 and GABRB3) were the first expressed sequences of known function assigned to the region (Wagstaff *et al.*, 1991a; Knoll *et al.*, 1993), and a high-resolution physical map of this $GABA_A$ receptor cluster has been constructed (Sinnett *et al.*, 1993).

Recently, Nakatsu *et al.* (1993) reported a deletion of $GABA_A$ receptor subunit genes $\alpha 5$, $\gamma 3$, and $\beta 3$ in mice with the pink-eyed cleft-palate (p^{cp}) mutation, with one breakpoint located in intron 1 of the p gene and the other in intron 3 of the $\beta 3$ gene. The assignment of the $\gamma 3$ receptor subunit gene to the p^{cp} deletion region on mouse chromosome 7 has been confirmed by others (Culiat *et al.*, 1994). The region on mouse chromosome 7 near the p locus shows a high degree of conservation of synteny with human chromosome 15q11–q13 (Chaillet *et al.*, 1991; Brilliant, 1992; Nicholls *et al.*, 1993; Brilliant *et al.*, 1994). The finding that the murine $\gamma 3$ subunit gene is tightly linked to the $\alpha 5$ and $\beta 3$ subunit genes suggests that the human homologue of the $\gamma 3$ gene could be within the $GABA_A$ receptor gene cluster on chromosome 15. We have confirmed this hypothesis and determined the transcriptional orientation of the human GABRG3 gene.

MATERIALS AND METHODS

Patient samples and cell lines. The following lymphoblastoid cell lines were used in this study: HS2, from a Prader–Willi patient with an interstitial deletion of chromosome 15q11–q13 (Nicholls *et al.*, 1989); GM0131, from a normal control; WJK106, from an Angelman patient with an unbalanced 13;15 translocation (45XY;−13, −15,+der[13]t[13;15][p13;q13]) (Knoll *et al.*, 1993).

DNA analysis. Genomic DNA was purified from cell lines according to standard procedures (Aldridge *et al.*, 1984). Two micrograms of DNA was digested with appropriate restriction enzymes (New England Biolabs, Boehringer Mannheim), and the fragments were separated by gel electrophoresis and transferred to Hybond N (Amersham). Blots were hybridized as described (Nicholls *et al.*, 1989). Genomic single-copy clones used for Southern blot hybridization were MN47 (D15S78, Buiting *et al.*, 1990), IR-10 (D15S12, Donlon *et al.*, 1986), and p53B (Sinnett *et al.*, 1993) from the PWS/AS chromosomal region and H2-26 from chromosome 13 (Lalande *et al.*, 1984). Hybridization probes derived from PCR products were amplified from genomic DNA or the appropriate phage clone and purified by gel electrophoresis. For quantitative analysis at least two experiments were carried out, and probe H2-26 was included as an internal standard.

Polymerase chain reactions. PCR conditions were 1× buffer as provided by Perkin Elmer, 1.5 m*M* $MgCl_2$, 0.125 m*M* each nucleotide, 0.5 μM primers, 1 U of *Taq* DNA polymerase (Perkin Elmer), and 5–25 ng of DNA in a 25-μl volume. PCR was carried out in a thermal cycler (Perkin Elmer) for 30 cycles of 30 s each at 94, 55, and 72°C. PCR products were separated on 3% agarose gels (NuSieve). PCR primers derived from the GABRG3 nucleotide sequence (Whiting *et al.*, in preparation) were as follows: 5′ end of the gene, 5′CAGCTTTTTATCATACTCTCTTAG3′ and 5′GATTCATCATCAAACCAAAAGTG3′; size of expected product, 110 bp; 3′ untranslated region, 5′TAAGTGTTGCTCAGAGTGAAGA3′ and 5′GTGTCCTTCCTTGCTACTCC3′ (103 bp). PCR primers derived from genomic phage clones were as follows: T3 end of P1 clone Π393, 5′GAAGAATGCCAACAAACAC3′ and 5′TGGCCACTATTTTTTCAAG3′ (89 bp); T7 end of P1 clone Π393, 5′GGCAGGAGGATTGTTTAAG3′ and 5′GGTTGTTTGTTTTTATGTGG3′ (239 bp); STS from phage λ189 near MN47, 5′GGCTTTGGGACCAGGTTATC3′ and 5′CATATTCCAACCAACATTCTGC3′ (186 bp). The primers from the 5′ and 3′ ends of GABRA5 and the dinucleotide polymorphism A5-39 have been described elsewhere (Knoll *et al.*, 1993; Glatt *et al.*, 1992). The chromosome 15 specificity of these PCR products was verified using DNA from a human (GM0131), a rodent (E36), and a rodent–human hybrid cell line containing chromosome 15 as the only human contribution (15A).

Sequencing. To generate sequence tagged sites (STSs) and probes for chromosome walking, the ends of λ and P1 phages were sequenced using the cycle sequencing kit from New England Biolabs according to the manufacturer's instructions. For sequencing of P1 clones, RNase A (0.1 mg/ml) was added to the reaction. Sequence analysis and PCR primers selection were performed with the primer analysis software oligo 4.0 (National Biosciences).

Library screening. Lambda phage clones were isolated by standard methods from a genomic library enriched for chromosome 15 sequences (Sinnett *et al.*, 1993). YAC clone ICRFy900H01126 was obtained from the Reference Library of the Imperial Cancer Research Fund, London (Lehrach *et al.*, 1990). High-density filters of a human YAC library were screened following the instructions with a PCR-amplified exon 9 probe of GABRB3 (Wagstaff *et al.*, 1991b). YAC ICRFy900H01126 was the largest (1.1 Mb) of a total of three positive clones. As tested by FISH analysis, this clone is nonchimeric. The STS from the 5′ end of GABRA5 was the most distal found in YAC ICRFy900H01126; therefore, the YAC contains the 5′ end of the $\alpha 5$ subunit and the complete $\beta 3$ subunit gene. P1 clones (Sternberg, 1992, 1994) isolated by PCR screening with the primer pairs from the 5′ and 3′ end of GABRG3 were obtained from Genome Systems, Inc. The original clone identification numbers are 1923, plate 377, well D3 for Π392; 1924, plate 983, well B4 for Π393; 1985, plate 406, well E7 for Π394; 1986, plate 406, well E10 for Π395; 1987, plate 1120, well G12 for Π396.

Fluorescence in situ hybridization (FISH). FISH was performed using large insert phage clone λ367 from GABRG3 (see text) as well as λ39 (previously called $\alpha 5$-39), which contains the 3′ untranslated region of GABRA5, and λ45 (previously called IR10-1-45), which was derived by screening a genomic phage library with probe IR-10 (Knoll *et al.*, 1993). λ367 was first localized to 15q11–q13 by hybridization to metaphase cells and was then included in the interphase map by simultaneous hybridization with phage clones λ39 and λ45 using methods as previously described (Knoll *et al.*, 1993).

RESULTS

Chromosomal and Subchromosomal Assignment

Primers derived from the sequence of the 3′ untranslated region of GABRG3 (Whiting *et al.*, in preparation) were used to perform PCR reactions with total human DNA, DNA from a hybrid cell line containing chromosome 15 as the only human contribution (A15), and DNA of the rodent parent of the hybrid (E36). The expected 103-bp product was observed in the human control and the hybrid cell line, but not in the rodent line, indicating that GABRG3 maps to chromosome 15 (data not shown). The subchromosomal location was determined by quantitative Southern blot hybridization, using radiolabeled PCR product obtained with the primers from the GABRG3 3′ end and DNA from a PWS patient (HS2) with a large 15q11–q13 deletion and an AS patient (WJK 106) with an unbalanced 13;15 translocation (45,XY;−13,−15,+der[13]t[13;15][p13;q13]). The breakpoint of this translocation has previously been mapped to intron 3 of the GABRB3 gene (Sinnett *et al.*, 1993). As shown in Fig. 1, the probe is hemizygously deleted in the DNA of patient HS2, but not in

FIG. 1. Mapping of GABRG3 by quantitative Southern blot hybridization. DNA samples of a normal control (C), a PWS patient with a large 15q11–q13 deletion (HS2), and an AS patient with an unbalanced 13;15 translocation resulting in a deletion of loci proximal to intron 3 of GABRB3 (WJK106) were digested with *Hin*dIII, separated by gel electrophoresis, and transferred to nylon membranes. Filters were probed with a PCR product amplified with primers from the 3′ untranslated region of GABRG3 and probe H2-26, a chromosome 13 marker used as internal hybridization standard. Only HS2 shows a signal of reduced intensity for the GABRG3 probe, indicating that GABRG3 maps within the PWS/AS chromosomal region, but distal to GABRB3.

that of patient WJK106 or a normal control. This result localizes GABRG3 within 15q11–q13, the region deleted in most PWS/AS patients, but distal to intron 3 of GABRB3.

Localization within the GABA Receptor Gene Cluster

The localization of the $\gamma 3$ subunit within the $GABA_A$ receptor gene cluster was initially determined by excluding the $\gamma 3$ subunit gene from the region between the $\beta 3$ and $\alpha 5$ subunits. A YAC (ICRFy900H01126) that comprises the entire $\beta 3$ and the 5′ portion of the $\alpha 5$ subunit gene was isolated from the ICRF library (Lehrach *et al.*, 1990) by screening with a probe from exon 9 of GABRB3. The primers from the 3′ end of GABRG3 did not yield an amplification product using the YAC DNA as a PCR template. Therefore, the STS is not contained in the YAC (data not shown). A library prepared from flow-sorted inv dup(15) chromosomes was screened with the PCR product of the $\gamma 3$ 3′ end, and three positive phage clones (λ367–369) were isolated. One of these clones (λ367) was utilized for interphase mapping by FISH. λ367, λ39 (containing the 3′ untranslated region of the $\alpha 5$ gene), and λ45 (D15S12) were simultaneously hybridized and detected by red and green fluorescence. An example of two-color/three-sequence ordering is given in Fig. 2. In 69 of 76 nuclei examined, GABRG3 maps between GABRA5 and D15S12. Starting with the most proximal gene, the order of the subunits in the GABA receptor gene cluster on chromosome 15 is, therefore, $\beta 3$–$\alpha 5$–$\gamma 3$.

Genomic Organization and Transcriptional Orientation

To investigate the genomic organization and to determine the transcriptional orientation of GABRG3, different approaches were used to isolate genomic clones for this gene. Since it seemed likely that GABRG3 is located in close proximity to GABRA5 based on the genomic organization of these two genes in the mouse (M. Brilliant, unpublished results), a phage walk beyond the 3′ end of this gene was initiated. Starting with p53B, a subclone of λ39, a set of three overlapping phage clones from the inv dup(15) library were obtained (Fig. 3a). Phage from each step were analyzed by PCR with primers from the 3′ and 5′ end of GABRG3 (Fig. 3b). Amplification with the 5′ end primer pair resulted in a 110-bp product in phage λ368, total human DNA, and the hybrid cell line A15, indicating that these phages contain the 5′ end of GABRG3. None of the phages was positive for the 3′ end primer pair. Since FISH analysis has placed the 3′ end of GABRG3 distal to GABRA5, these results establish the transcriptional orientation of GABRG3. Both genes are transcribed in the same orientation, with the 5′ end located closer to the centromere and the 3′ end pointing toward the telomere. As shown by the restriction map in Fig. 3a, the 3′ untranslated region of GABRA5 and the first exons of GABRG3 are less than 35 kb apart, which is very similar to the organization in the mouse (Brilliant, unpublished).

The primer pairs from both ends of GABRG3 were also used to screen a P1 library (Sternberg, 1992). Three clones for the 5′ end (Π394–396) and two clones for the 3′ end (Π392–393) were isolated and analyzed with a combination of restriction mapping, fingerprinting by inter-*Alu* PCR, and STS mapping. The results of these experiments are summarized in Figs. 3a and 4. Clones Π392 and Π393 from the 3′ end of GABRG3 are 64 and 87 kb in size, and restriction mapping revealed that the smaller clone is contained within the larger one. Except for the STS originally used for screening, these clones were negative for all STSs tested. Hybridization experiments showed that these clones do not contain the marker IR10 from the human P locus. Clones Π395 and Π396 from the 3′ end of GABRG3 are 55 and 82 kb in size; clone Π394 turned out to be identical with Π395 and was not analyzed further. As shown in the map (Fig. 3a), both clones overlap by approximately 35 kb, with Π395 extending further into the GABRG3 gene and Π396 comprising the 3′ end of GABRA5 in addition to the first exons of GABRG3. Restriction mapping and fingerprinting by inter-*Alu* PCR gave no indication of a physical link between the two sets of P1 clones from the respective ends of GABRG3. Therefore, the ends of Π393 were sequenced, and selected primers defining a STS at each end of the clone were designed. None of the P1 clones from the 5′ end was positive for either STS, confirming that clones from different ends of the gene do not overlap. Π395 contains 33 kb of the 5′ portion, and Π393 contains a minimum of 20 kb of the 3′ portion of GABRG3 (the available data do not allow the orientation of Π393, and for this reason the exact size cannot be determined), indicating that the GABRG3 gene spans more than 50 kb.

FIG. 2. Interphase mapping of GABRG3 by fluorescence *in situ* hybridization. Interphase mapping of GABRG3 was accomplished by simultaneous hybridization with differentially labeled λ39 and λ45. λ367 was labeled with digoxigenin–dUTP and detected with rhodamine-conjugated antibody to digoxigenin; λ39 and λ45 were labeled with biotin–dUTP and detected with fluorescein-labeled avidin. Two independent hybridization experiments yielded the order λ39, λ367, and λ45 (green, red, green). Cells were counterstained with DAPI. Cells were imaged with a Zeiss-Axiophot epifluorescence microscope equipped with a cooled charge-coupled device camera (Photometrics) operated by a Macintosh Quadra computer. DAPI, rhodamine, and fluorescein images were captured individually in gray scale using the appropriate filters and IP Lab software. The images were then merged and pseudocolored by IP Lab software. Residual fluorescein accounts for the single bright green fluorescent spot in the right nucleus (at 10 o'clock).

Assignment of MN47 to a New Location on the Physical Map

The P1 clones were also analyzed with STS 189, which was derived from a phage (λ189) isolated with microclone MN47 (Buiting *et al.*, 1990). The STS was present only in Π396, the clone containing parts of the α5 subunit gene. MN47 has previously been placed 100 kb distal of the GABRA5 gene by PFGE mapping (Sinnett *et al.*, 1993), a result contradictory to our finding. Reevaluation of a phage contig (represented by λ189 and λ350) surrounding MN47 revealed that it overlaps with the phage contig spanning the 3′ end of the GABRA5 gene. The correct position of MN47 is about 7 kb proximal to the 3′ UTR of GABRA5. Since MN47 is evolutionarily conserved and located within the GABRA5 gene, we considered the possibility that it contains exon sequences of GABRA5. However, there was no significant sequence homology between the GABRA5 cDNA and MN47.

DISCUSSION

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in mammalian brain; therefore, abnormalities in $GABA_A$ receptor gene structure or function could play an important role in neurological diseases. GABRB3 was the first subunit gene to be localized to chromosomal subregion 15q11–q13 (Wagstaff *et al.*, 1991a), which is frequently deleted in Angelman syndrome. This genetic disorder is characterized by seizures, severe developmental delay, ataxic gait, jerky movements, microcephaly, and outbursts of inappropriate laughter (Clayton-Smith and Pembrey, 1992). Perturbation of the interaction between GABA and its receptor complex could result in some of the neurological manifestations of AS. A second subunit gene, GABRA5, was subsequently assigned to this region (Knoll *et al.*, 1993). To determine its genomic organization, the region surrounding the two genes was mapped by PFGE and genomic phage cloning (Sinnett *et al.*, 1993). As demonstrated here, a third subunit gene, GABRG3, maps to this receptor cluster in 15q11–q13.

The construction of the high-resolution map of the β3, α5, and γ3 subunit cluster by generating a λ and P1 phage contig permitted localization of the 5′ end of the γ3 gene with respect to the 3′ end of the α5 gene and the determination of the transcriptional orienta-

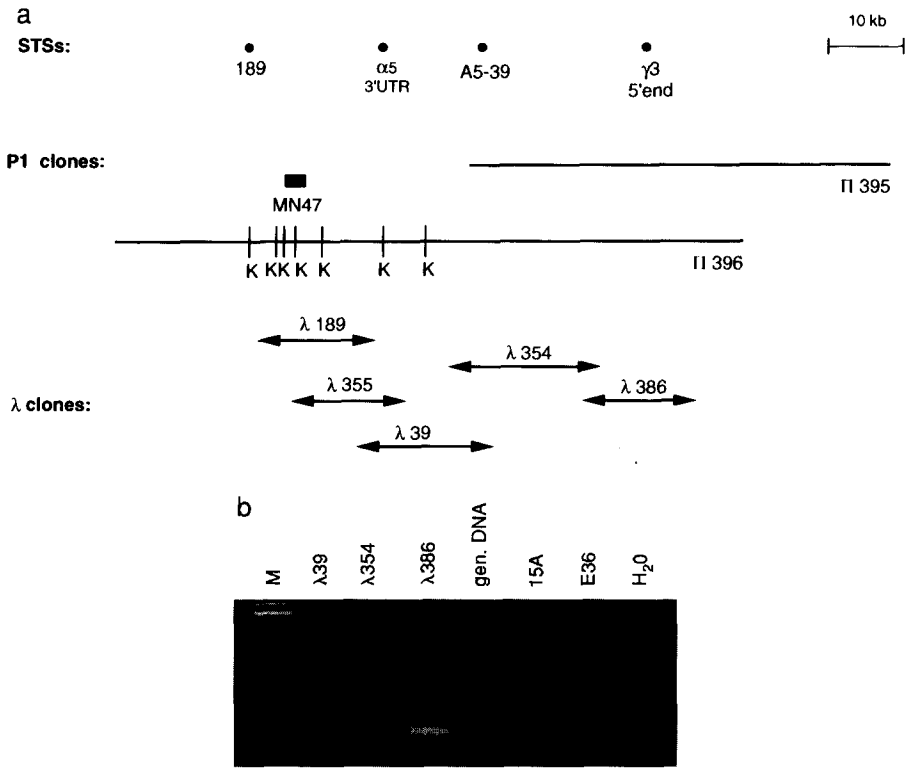

FIG. 3. The region surrounding the 3′ end of the α5 subunit and 5′ end of the γ3 subunit gene. (**a**) Map of P1 and λ phage clones. Clones were analyzed by restriction and STS mapping. STSs used are shown as dots in the first line. K, *Kpn*I recognition site. The corresponding *Kpn*I restriction sites were found in λ phage clones (not shown). The marker MN47 is represented by a shaded rectangle. (**b**) Example of STS analysis. Clones from the phage walk beyond the 3′ end of GABRA5 (compare **a**) were analyzed by PCR with the primer pair from the 5′ end of GABRG3. Samples of total genomic DNA, DNA from a hybrid cell line containing chromosome 15 as the only human contribution (A15), and DNA from the rodent parent (E36) were included as controls.

tion. The α5 and the γ3 subunit genes have the same transcriptional orientation and lie less than 35 kb apart, while the α5 and the β3 subunit genes are transcribed in opposite directions, positioned in head to head configuration, and separated by approximately 100 kb. The γ3 subunit spans at least 50 kb, but since the genomic phage contigs surrounding the ends of the gene have not yet been linked, it may turn out to be substantially larger. Together, the three genes extend over more than 500 kb.

Close conservation of synteny exists between the $GABA_A$ cluster on human chromosome 15 and its homologous counterpart on the murine chromosome 7 (Chaillet *et al.*, 1991). Mice homozygous for the p^{cp} mutation, a deletion that encompasses the murine α5 and γ3 and part of the β3 subunit genes, display tremor and a jerky gait, neurological malfunctions reminiscent of AS (Nakatsu *et al.*, 1993; Culiat *et al.*, 1994). These findings could suggest that deletions within the β3, α5, and γ3 subunit cluster are associated with the AS phenotype. In humans, however, hemizygous (maternal) deletions lead to AS, while only homozygous deletions cause a recognizable phenotype in mice. If imprinting in this region is conserved in rodents, these data would argue against the involvement of these receptor genes in AS. In addition, the genes in the $GABA_A$ receptor cluster on 15q11–q13 have been excluded from the smallest region of deletion overlap in this syndrome (Reis *et al.*, 1993; Buxton *et al.*, 1994). The latter data imply either that the β3, α5, and γ3 subunit genes

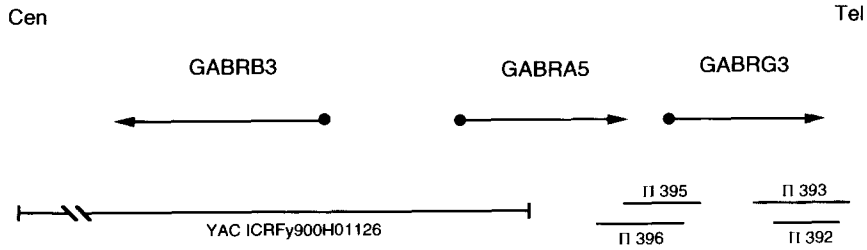

FIG. 4. The $GABA_A$ receptor cluster on chromosome 15q11–q13. The β3, α5, and γ3 subunit genes are symbolized by arrows that indicate the direction of transcription. The position of YAC and P1 clones from the region is given below. The map is not drawn to scale.

play no role in the development of AS or that chromosomal rearrangements occurring outside the $GABA_A$ receptor cluster can disrupt imprinting in this region. There is evidence to suggest that the human $GABA_A$ receptor cluster is subject to imprinting, since different patterns of allele-specific DNA replication timing are observed in the cluster (Kitsberg *et al.,* 1993; Knoll *et al.,* 1994). It is not clear, however, that this parental origin-dependent effect on replication is related to allele-specific gene expression.

The clustering of other $GABA_A$ receptor α, β, and γ subunit genes has been described at two other locations in the human genome: for $\alpha 2$, $\beta 1$, and $\gamma 1$ on chromosome 4p12–p13 (Dean *et al.,* 1991; Kirkness *et al.,* 1991; Wilcox *et al.,* 1992) and $\alpha 1$, $\alpha 6$, $\beta 2$, and $\gamma 2$ on chromosome 5q34–q35 (Wilcox *et al.,* 1992; Hicks *et al.,* 1994; Russek and Farb, 1994). Analogous to our findings for $\alpha 5$ and $\gamma 3$, Wilcox *et al.* (1992) report that the $\alpha 1$ and $\gamma 2$ subunits are located within 200 kb of each other. Although the available data are too limited to draw any conclusions, it seems possible that different $\alpha/\beta/\gamma$ clusters are structured in a similar fashion. It has been postulated that the different subunit genes derive from a common ancestor and spread throughout the genome during evolution. A similar arrangement of genes within these clusters would exclude simple duplication events and require the transposition of large stretches of DNA. Comparison of the organization of the clusters will reveal their relationship to each other and may provide insight into their evolutionary origin.

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