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Chromosomal localization of the human diazepam binding inhibitor gene

(gene mapping/*in situ* hybridization/somatic cell hybrids)

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ABSTRACT **We have used *in situ* chromosome hybridization and human–mouse somatic cell hybrids to map the gene(s) for human diazepam binding inhibitor (DBI), an endogenous putative modulator of the γ-aminobutyric acid receptor acting at the allosteric regulatory center of this receptor that includes the benzodiazepine recognition site. In 784 chromosome spreads hybridized with human DBI cDNA, the distribution of 1476 labeled sites revealed a significant clustering of autoradiographic grains (11.3% of total label) on the long arm of chromosome 2 (2q). Furthermore, 63.5% of the grains found on 2q were located on 2q12–21, suggesting regional mapping of DBI gene(s) to this segment. Secondary hybridization signals were frequently observed on other chromosomes and they were statistically significant mainly for chromosomes 5, 6, 11, and 14. In addition, DNA from 32 human–mouse cell hybrids was digested with *Bam*HI and probed with human DBI cDNA. A 3.5-kilobase band, which probably represents the human DBI gene, was assigned to chromosome 2. Four higher molecular weight bands, also detected in *Bam*HI digests, could not be unequivocally assigned. A chromosome 2 location was excluded for the 27-, 13-, and 10-kilobase bands. These results assign a human DBI gene to chromosome 2 (2q12–21) and indicate that three of the four homologous sequences detected by the human DBI probe are located on three other chromosomes.**

Diazepam binding inhibitor (DBI), a 10-kDa peptide, is an endogenous modulator of the γ-aminobutyric acid ($GABA_A$) receptor acting at the allosteric regulatory center of this receptor that includes the benzodiazepine recognition site (1–4).

The human, rat, and bovine DBI complementary DNAs have been isolated, cloned, and sequenced (5–7). Hybridization of the DBI cDNA to genomic DNA suggests that DBI is encoded by a multigene family of approximately five members (5, 6). The functional significance of these genomic bands hybridizing with the DBI cDNA is not clear at present and, although a single gene appears to account for the DBI mRNA present in tissues, it cannot be ruled out that more than one active gene exists.

DBI and its processing products have been characterized biochemically, behaviorally, and electrophysiologically for their negative modulatory action on the $GABA_A$ receptor (3, 4, 8, 9). Moreover, *in vivo* studies have shown that DBI and its processing products have the pharmacological profile of a naturally occurring anxiogenic compound (2, 8, 9). Further investigations, however, are needed to fully understand the neurobiological significance of DBI and its processing products.

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Another fascinating and still unexplored line of research concerns the potential role of DBI in the pathogenesis of neurological disorders. Because of its increased content in cerebral spinal fluid of severely depressed patients (10), DBI might participate in the pathogenesis of depressive psychosis and severe anxiety. Recently, molecular biology techniques combined with more refined methods of cytogenetics have led to the discovery of a relationship between expression of a particular gene and pathogenesis of certain neurological diseases. A starting point in establishing such a relationship is the chromosomal location of the gene being studied. The use of DBI cDNA probe allowed us to take the first step toward understanding the molecular genetics of this neuropeptide. We report that a human DBI gene is located on chromosome 2 (2q12–21) as a result of *in situ* hybridization studies and Southern blot analysis of human–mouse hybrid cell lines.

MATERIALS AND METHODS

Chromosome Preparation. Human chromosome spreads were prepared from phytohemagglutinin-stimulated methotrexate-synchronized peripheral blood lymphocytes of normal donors by standard techniques (11, 12). Chromosome slides were used within 4 weeks for *in situ* hybridization experiments.

DNA Probe. The 415-base-pair *Eco*RI DNA fragment, containing a partial sequence of DBI cDNA, was isolated from pHu22A-DBI plasmid (a gift from P. Gray, Genentech, South San Francisco, CA) (5). Labeling of this fragment was carried out by the random primer extension procedure as described by Feinberg and Vogelstein (13), with 0.75 nmol each of [^{3}H]dATP (40.1 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]dTTP (92.2 Ci/mmol), and [^{3}H]dCTP (53.3 Ci/mmol) (New England Nuclear). The DNA, labeled to a specific activity of 2×10^7 cpm/μg, was separated from free [^{3}H]dNTP by centrifugation through 1 ml of hydrated Sephadex G-50 at $1800 \times g$.

***In Situ* Hybridization.** *In situ* hybridization was performed by a modification of the technique described by Harper *et al.* (14). Air-dried chromosome preparations were treated with RNase A (Boehringer Mannheim) at 100 μg/ml in 2× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0) at 37°C for 1 hr, rinsed in 2× SSC, and dehydrated through an alcohol series. The chromosomes were denatured in 70% (vol/vol) formamide/2× SSC, pH 7.0, at 70°C for 2 min, quickly dehydrated in an alcohol series, and then air-dried. The hybridization mixture, containing the ^{3}H-labeled DNA probe (0.1–0.5 μg/ml), 10% dextran sulfate, 50% formamide, 2× SSC, 40 mM sodium phosphate (pH 7.0), and a 500- to

Abbreviation: DBI, diazepam binding inhibitor.

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Table 1. Hybridization of human DBI probe to metaphase chromosomes

Exp.	No. of metaphases	No. of grains	No. of grains on 2q	No. of grains on 2q12–21
1	244	322	30/322 (9.3%)	11/322 (3.4%)
2	80	170	21/170 (12.3%)	13/170 (7.6%)
3	50	70	9/70 (13.0%)	6/70 (8.6%)
4	193	348	30/348 (8.6%)	17/348 (5.0%)
5	217	566	77/566 (13.6%)	59/566 (10.4%)
Total	784	1476	167/1476 (11.3%)	106/1476 (7.2%)
			$\chi^2 = 130$	$\chi^2 = 440$
			$P < 0.001$	$P < 0.001$

Exps. 1 and 2, lower stringency experiments; exps. 3–5, higher stringency experiments.

1000-fold excess of sonicated salmon sperm DNA was denatured at 70°C for 5 min and quickly cooled on ice. Eighty microliters of this mixture (10–50 ng probe DNA; 1–5 × 10^5 cpm) was placed on each slide and the hybridization was carried out at 37°C for 16–24 hr. For the lower stringency posthybridization washing, the slides were rinsed three times, 2 min each, in 50% formamide/2× SSC, pH 7.0, at 39°C, and then five times, 2 min each, in 2× SSC at 39°C. For the higher stringency experiments, the washes were done in 50% formamide/2× SSC and 2× SSC, respectively, three times, 5 min each, at 42°C, and then in 1× SSC five times, 2 min each, at 42°C and, finally, in 1× SSC three times, 15 min each, at room temperature. Thereafter, the slides were dehydrated through an alcohol series and air-dried. Preparations were exposed to Kodak NTB_2 nuclear track emulsion (Eastman) for 10–21 days at 4°C, then developed in Kodak D19, fixed, and thoroughly rinsed in distilled water. The hybridized chromosomes were G-banded as described by either Harper *et al.* (14) or Cannizzaro and Emanuel (15). Analysis of the slides was carried out without knowledge of the results from the human–mouse hybrid studies. Metaphase spreads with good chromosome morphology and limited background grains were selected. Only grains lying over or in contact with a chromatid were scored; clusters of grains found at the same site were considered as a single hybridization event. The location of specific grains was recorded on ISCN (International System for Human Cytogenetic Nomenclature) ideograms (16) and the observed distribution of grains over each chromosome arm was compared by χ^2 analysis to a random distribution expected on the basis of the relative length of each arm (17).

Hybrid Cell Lines and Southern Blot Analysis. Construction and characterization of the human–mouse cell lines have been described (18–21). A total of 32 cell hybrids involving 16 unrelated human and 4 mouse cell lines were used. Human chromosomes were identified in cell hybrids by the Giemsa–trypsin banding technique. On the same passage, cell hybrids were examined for 31 human chromosome-specific enzyme markers to confirm the chromosome analysis.

DNA (10 μg) from each cell line was digested with *Bam*HI and electrophoresed in 0.8% agarose gels at 35 V in TAE buffer (40 mM Tris/20 mM acetic acid/2 mM EDTA, pH 8.1). The DNA was transferred to Zetapore nylon membranes (Cuno, Meriden, CT) according to the manufacturer's instructions and covalently bound by UV light (22). Human DBI cDNA probe, the same as that used for the *in situ* hybridization experiments, was labeled with [^{32}P]dCTP (3000 Ci/mmol) (Amersham) by the primer extension method (13) to a specific activity of 6.7 × 10^5 cpm/ng. Blots were hybridized with 8 ng of DNA probe per blot for 24 hr in 50% formamide at 42°C and given three 5-min low stringency washes (2× SSC/0.1% $NaDodSO_4$) at room temperature and one 10-min high stringency wash (0.1× SSC/0.1% $NaDodSO_4$) at 50°C. Blots were exposed for 7 days with Kodak XAR-5 film and Lightning Plus Image screen at −80°C. The interpretation of the autoradiographs was carried out without knowledge of both the chromosomal make-up of the individual cell lines and the results from the *in situ* hybridization studies.

RESULTS

***In Situ* Hybridization Studies.** To determine the chromosomal location of the human DBI gene(s), five independent experiments were performed. The efficiency of hybridiza-

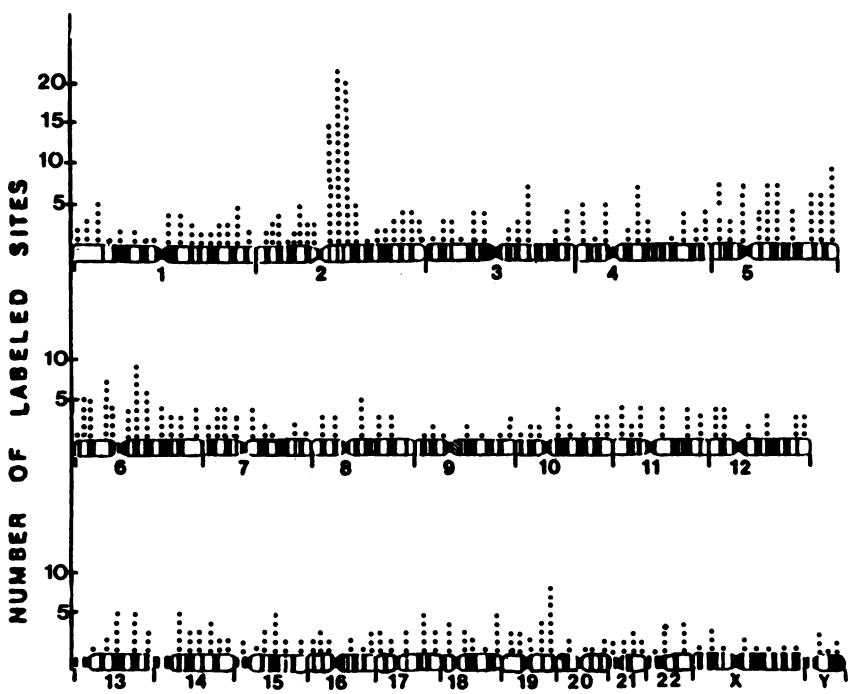

FIG. 1. Diagram showing grain distribution in 217 metaphase spreads. The distribution of 566 grains was scored, 59 (10.4%) were found overlying the 2q12–21 segment.

tion, previously tested by using single copy sequences of known chromosomal assignment, was evaluated by examining 784 metaphase spreads that yielded 1476 autoradiographic grains (an average of 1.9 grains per cell). In the first two experiments, in which a lower stringency posthybridization washing procedure was applied, 324 cells were analyzed with a total of 492 grains; 15.7% of the metaphases exhibited label on the long arm of chromosome 2 (2q). Of the 51 grains (10.4% of total labeled sites) observed on 2q, 24 were located on 2q12–21 segment. For the higher stringency experiments, 460 cells were scored with a total of 984 silver grains. Consistent with the first observation, the predominant site of hybridization (25.2% of the mitosis) was the long arm of chromosome 2 displaying 11.8% of the total grain count. Moreover, of the 116 labeled sites observed on 2q, 82 (70.7%) were located on 2q12–21 region. The results of the individual experiments are presented in Table 1. Combining both sets of experiments, the long arm of chromosome 2 was found labeled in 21.3% of the spreads analyzed, exhibiting 11.3% of the total grain count. The deviation from the expected number of hybridization events on 2q, calculated on the basis of proportionality to chromosome arm length (17), was highly significant ($\chi^2 = 130$; $P < 0.001$). Furthermore, 63.5% (106/167) of the grains observed on 2q were located on 2q12–21 region, representing 7.2% of the total label. Again, the deviation from the expected number of grains on 2q12–21, which represents ≈1.2% of the haploid genome, was highly significant ($\chi^2 = 440$; $P < 0.001$). The results from a representative experiment (exp. 5) are illustrated in Fig. 1, where the distribution of 566 labeled sites from 217 hybridized cells shows significant clustering of grains on 2q12–21. Typical chromosomal spreads with silver grains on 2q12–21 are presented in Fig. 2. The sublocalization of DBI gene(s) on 2q is illustrated in Fig. 3, where the grain distribution of 100 labeled chromosomes 2 reveals a concentration of grains on region 2q12–21. These results suggest regional mapping of a DBI gene to the segment 2q12–21. However, secondary peaks of hybridization on chromosomes other than number 2 were frequently observed. In at least one of the two lower stringency experiments, statistically significant clustering of grains was observed on the long arm of chromosomes 1, 5, 14, and 15 and on the short arm of chromosome 11. When higher stringency conditions were applied, the long arm of chromosome 5 was again found significantly labeled in two of three experiments and additional peaks of hybridization were observed on other chromosomes. In particular, the long arm of chromosomes 6, 14, 19, and 20, and the short arm of chromosomes 11 and 17 exhibited grains significantly in excess of the expected number in at least one of the experiments performed with a more stringent washing procedure. However, unlike what was observed on chromosome 2, on most of the other chromosomes the labeling was distributed rather randomly on their various arm regions.

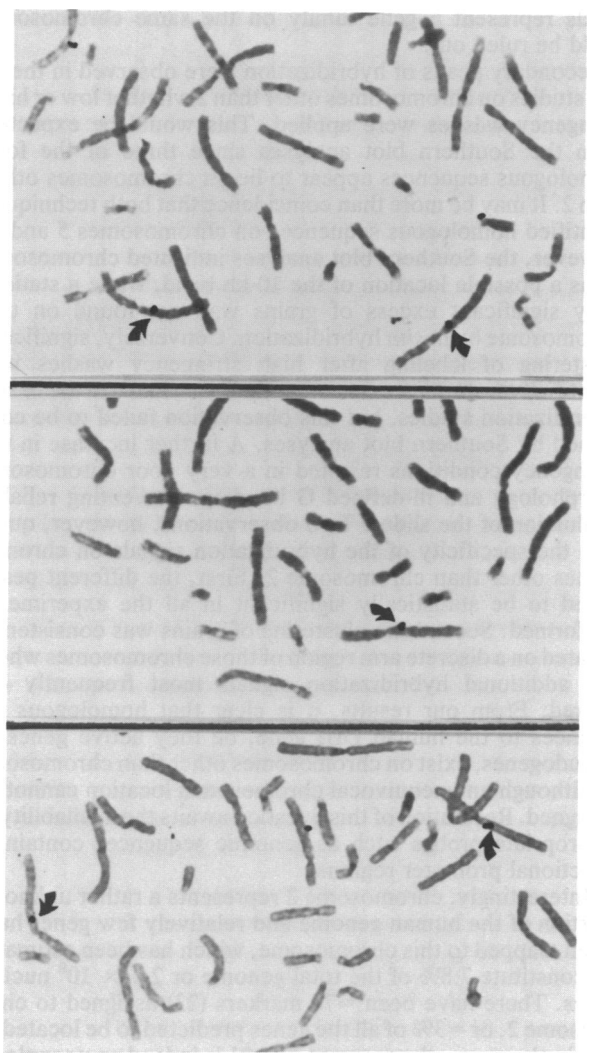

FIG. 2. Three partial human metaphases hybridized with ^{3}H-labeled cDNA for DBI at a concentration of 0.2 μg/ml, illustrating typical labeling of q12–21 region of the long arm of chromosome 2 (arrows).

Somatic Cell Hybrid Analyses. The human DBI cDNA probe detected five nonpolymorphic bands in human genomic DNA digested with *Bam*HI [27, 21, 13, 10, and 3.5 kilobases (kb)], as shown by Gray *et al.* (5). The 3.5-kb band was more intense than the other four and, therefore, probably corresponds to an active DBI gene. Two bands were detected in mouse DNA (17 and 4.9 kb) that were distinguishable from all five human bands. In an analysis of DNA from 32 human–mouse hybrid cell lines, the 3.5-kb DBI band segregated with chromosome 2 (Table 2; Fig. 4). The other bands were more difficult to interpret because of their fainter intensity, and a definite chromosomal localization could not be established. However, chromosome 2 can probably be excluded for the

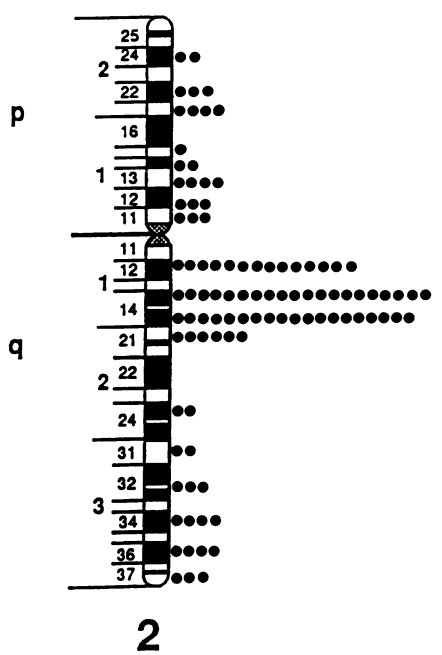

FIG. 3. Distribution of silver grains on 100 labeled chromosomes 2. Of the 77 grains observed on 2q, 59 (76.6%) are located on q12–21 region.

Table 2. Segregation of human DBI with human chromosomes in human–mouse hybrid cell lines

DBI band	Concordancy	Human chromosome																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
3.5 kb	+/+	8	14	10	9	10	10	11	9	7	12	8	11	9	11	9	7	11	10	8	9	11	7	8
	−/−	16	18	10	13	11	15	10	8	15	6	13	10	12	6	10	13	7	11	14	6	5	9	7
	+/−	6	0	3	5	4	4	2	5	7	2	4	3	5	3	4	7	2	4	6	5	3	6	3
	−/+	1	0	7	5	7	3	7	10	1	12	4	8	6	12	8	5	11	7	4	12	13	8	7
Discordancy (%)		23	0	33	31	34	22	30	47	27	44	28	34	34	47	39	37	42	34	31	53	50	47	40

Concordant hybrids (+/+ and −/−) have either retained or lost the specified DBI band together with a specific human chromosome. Discordant hybrids (+/− and −/+) either retained the genes but not a specific chromosome (+/−), or the reverse (−/+). Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment. Results are based on a total of 32 mouse–human hybrid cell lines analyzed. Totals are <32 in some cases because of the exclusion of cell lines containing translocated chromosomes.

27-, 13-, and 10-kb bands since the discordancies between the presence and absence of bands and the presence and absence of chromosome 2 ranged from 32% to 38%. The discordancy for the 21-kb band was 19% (4/21), leaving chromosome 2 as a possible assignment, although the same percentage of discordancy was found for chromosomes 12 and 18.

DISCUSSION

In situ hybridization technique and Southern blot analysis of human–mouse somatic cell hybrids have been used to determine the chromosomal localization of the human DBI gene. Since five homologous sequences per haploid genome are identified in Southern blots after hybridization with human DBI cDNA, one might expect more than one chromosomal site to be detected by *in situ* hybridization. The analysis of 1476 autoradiographic grains from 784 metaphase spreads showed a statistically significant ($P < 0.001$) clustering of labeled sites on the q12–21 region of the long arm of chromosome 2. The grains located along this region almost doubled in number (from 4.8% to 8.3% of all chromosomal label) when higher stringency posthybridization washes were performed. From our results, however, a more precise chromosomal band assignment of the DBI gene could not be established because of the rather wide distribution of grains along the 2q12–21 segment, which is a relatively large region of the long arm of chromosome 2. A further resolution of the gene's location within the 2q12–21 region could be achieved by using cell lines cytogenetically selected for carrying chromosomal rearrangements involving such a region.

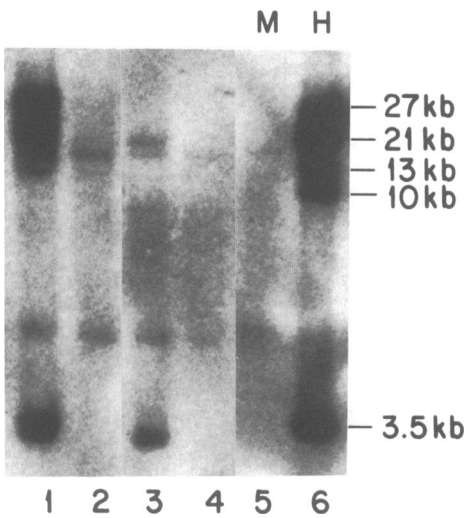

FIG. 4. Southern blot of mouse–human hybrid cell lines showing cosegregation of the 3.5-kb human DBI band with human chromosome 2. Lanes 1 and 3, human chromosome 2 is present; lanes 2 and 4, human chromosome 2 is absent. Lane 5, mouse genomic DNA; lane 6, human genomic DNA.

The results of *in situ* hybridization studies were complemented by the Southern blot analyses of human–mouse hybrid cell lines. The 3.5-kb *Bam*HI band, which probably represents a human DBI gene by virtue of the cDNA probe preferentially hybridizing to this band, was assigned to chromosome 2. By contrast, three of the four high molecular weight bands could be excluded from chromosome 2. Even though unequivocal assignments could not be made for the high molecular weight bands, the possibility that the five bands represent a gene family on the same chromosome could be ruled out.

Secondary peaks of hybridization were observed in the *in situ* studies on chromosomes other than 2 whether low or high stringency washes were applied. This would be expected from the Southern blot analyses since three of the four homologous sequences appear to lie on chromosomes other than 2. It may be more than coincidence that both techniques identified homologous sequences on chromosomes 5 and 6; however, the Southern blot analyses indicated chromosome 16 as a possible location of the 10-kb band, while a statistically significant excess of grains was not found on this chromosome by *in situ* hybridization. Conversely, significant clustering of labeling after high stringency washes was identified on chromosomes other than 2, 5, and 6 in the *in situ* hybridization studies, but this observation failed to be confirmed by Southern blot analyses. A further increase in the stringency conditions resulted in a very poor chromosome morphology and ill-defined G banding, preventing reliable evaluation of the slides. Two observations, however, question the specificity of the hybridization signals on chromosomes other than chromosome 2. First, the different peaks failed to be statistically significant in all the experiments performed. Second, no clustering of grains was consistently located on a discrete arm region of those chromosomes where the additional hybridization signals most frequently occurred. From our results, it is clear that homologous sequences to the human DBI gene, be they active genes or pseudogenes, exist on chromosomes other than chromosome 2, although an unequivocal chromosomal location cannot be assigned. Resolution of this question awaits the availability of appropriate probes such as genomic sequences containing functional promoter regions.

Interestingly, chromosome 2 represents a rather unknown portion of the human genome and relatively few genes have been mapped to this chromosome, which has been estimated to constitute 7.8% of the total genome or 2.3×10^8 nucleotides. There have been ≈70 markers (23) assigned to chromosome 2, or ≈3% of all the genes predicted to be located on it. Furthermore, the segment q12–21 is indeed an unexplored region within chromosome 2, with only one gene, interleukin 1, having been mapped at q13–21 (23). The localization of the DBI gene, together with future studies on restriction fragment length polymorphisms linked to it, should improve our

knowledge of the genetic and functional significance of chromosome 2.

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