

INTRODUCTION

Twin, family, and adoption studies have consistently revealed that genetic factors are involved in Schizophrenia (Gottesman, 1997; Moldin and Gottesman, 1997; Kirov and Murray, 1997; Tsuang, 1998). The disease is familial, with risks in relatives that are many times greater than the population average, which is 1 percent. Furthermore, the risk parallels the proportion of genes shared with the affected relative. For example, the rate of schizophrenia among first-degree biological relatives of individuals with schizophrenia is approximately 10%, while the risk to second-degree biological relatives is between 2~6%. Moreover, concordance rates for schizophrenia are higher in monozygotic twins (approximately 50%) compared to dizygotic twins (approximately 17%). Adoption studies have shown that biological relatives of individuals with schizophrenia have a substantially increased risk for schizophrenia, whereas adoptive relatives do not display an increased risk. While it is certain that genetic factors play an important role in the etiology of schizophrenia, the existence of a substantial discordance rate in monozygotic twins suggests that environmental factors may also be important (Moldin and Gottesman, 1997; Tsuang, 1998). It is likely that genetic and environmental risk factors interact in a complex fashion to produce the disease. For example, certain genes may function to alter an individual's sensitivity to environmental factors that are of etiological relevance to the disease (Moldin and Gottesman, 1997).

Identifying genes that effect an individual's predisposition to develop schizophrenia could improve diagnosis of the disease, and perhaps lead to improvements in prevention and in pharmacological treatment of the disease. Two main approaches have been used in the search for such genes: genetic linkage studies, and genetic association studies of candidate genes. A number of recent linkage studies have identified potential susceptibility loci on chromosomes 6p, 8p, 9, 20 and 22 (Antonarakis *et al.*, 1995; Moises *et al.*,

1995; Schwab *et al.*, 1995; Straub *et al.*, 1995; Wang *et al.*, 1995; Schizophrenia linkage collaborative group for chromosomes 3, 6 and 8, 1996; Turecki *et al.*, 1997). However, other reports have produced results that contradict these studies. This has led some to conclude that susceptibility to schizophrenia may be largely due to an interaction between several genes, each of which produce relatively small effects on disease risk (Levinson *et al.*, 1998; Shaw *et al.*, 1998). On the other hand, evidence from other experimental approaches, including pharmacology, suggests the importance of the dopaminergic and/or serotonergic system in schizophrenia (Breier, 1995; Iqbal and Praag, 1995; Willner, 1997; Kawanishi and Suzuki, 1999). This evidence has given rise to numerous association studies of genes that encode the dopamine and serotonin receptors. Note however, that some of the genetic variants of these receptors are not clearly associated with schizophrenia. Though there have been reports of positive associations for certain other variants, none of these results has been replicated consistently (Yang *et al.*, 1993; Arinami *et al.*, 1994; Cichon *et al.*, 1994; Daniels *et al.*, 1994; Erdmann *et al.*, 1995; Sobell *et al.*, 1995; Cichon *et al.*, 1996; Crawford *et al.*, 1996; Erdmann *et al.*, 1996; Verga *et al.*, 1997; Kawanishi and Suzuki, 1999).

Environmental effects including experience, nonspecific stressors, and drugs can elicit long-term and plastic changes in the function of the brain (Hyman and Nestler, 1993). Afferent sensory data activate particular neural networks in the brain, which in turn activate neurons involved in higher-order processing. Within each of the cells involved in these networks, the generation of action potentials and the activation of second messenger systems would be expected to alter the rate of expression of specific genes. This in turn would lead to changes in the expression of multiple types of neuronal proteins. Altered levels of these proteins would produce characteristic changes in the way the affected neurons process subsequent synaptic information. The process by which activity in one neuron regulates gene expression in another

neuron is referred to as transsynaptic regulation of gene expression (Hyman and Nestler, 1993). Neurotransmitters, acting through cell surface receptors linked to the activation of second messenger systems, can regulate gene expression (Comb *et al.*, 1987). Transsynaptic regulation of gene expression is one of the molecular mechanisms underlying the long-term and plastic effects of environmental factors on both the developing and adult brain (Comb *et al.*, 1987; Hyman and Nestler, 1993).

Schizophrenia has plastic aspects. The relationship between genetic and environmental etiological factors may take the form of an interaction between the environment and the brain's own mechanisms for adaptive and plastic change (Haracz, 1984; Haracz, 1985). Such an interaction may be of relevance to the age of onset of the disease (i.e. that does not occur soon after birth), the repetition of the relapses and remissions that characterize its longitudinal course, and modulatory influences of the environment on the course of schizophrenia (Haracz, 1984; Haracz, 1985; Kaplan and Sadock, 1998). This suggests that dysfunction in the transsynaptic regulation of gene expression could play a role in the disease. Many genes, including receptors, messenger molecules of intracellular signal-transduction pathways, and transcription factors are involved in transsynaptic regulation of gene expression. Some of these genes have also been implicated in schizophrenia.

Dysfunction in the serotonergic system may also contribute to schizophrenia (Breier, 1995; Iqbal and Praag, 1995). More specifically, attention has focused on the 5-hydroxytryptamine 1A (5-HT_{1A}) receptor in studies of schizophrenia, particularly in psychopharmacological fields. This interest followed the observation that clozapine has an appreciable affinity for the 5-HT_{1A} receptor (Mason and Reynolds, 1992), while the 5-HT_{1A} receptor agonist buspirone displays antipsychotic activity when given as an adjunct to neuroleptic therapy (Goff *et al.*, 1991). Moreover, a number of authors have reported that 5-HT_{1A} receptor is increased in the postmortem prefrontal cortex

of schizophrenic patients (Hashimoto *et al.*, 1991; Hashimoto *et al.*, 1993; Burnet *et al.*, 1996; Simpson *et al.*, 1996; Sumiyoshi *et al.*, 1996). These reports suggest that schizophrenia may involve an alteration in the expression of the 5-HT_{1A} receptor in the central nervous system. Recent studies at the molecular level have identified a number of 5-HT_{1A} receptor gene variants including Pro16Leu, Gly22Ser, Ile28Val, 294G→A, 549C→T, Arg219Leu and Asn417Lys (Erdmann *et al.*, 1995; Nakhai *et al.*, 1995; Xie *et al.*, 1995; Harada *et al.*, 1996; Lam *et al.*, 1996). No associations have been found between the variants (Ile28Val, 294G→A) and schizophrenia (Erdmann *et al.*, 1995; Inayama *et al.*, 1996). Further study is required in order to determine if other genetic variants in the coding and/or promoter region of the 5-HT_{1A} receptor gene confer a predisposition towards schizophrenia.

Many recent linkage studies have suggested that the susceptibility loci of schizophrenia is present on chromosome 6p24-21 (Antonarakis *et al.*, 1995; Moises *et al.*, 1995; Schwab *et al.*, 1995; Straub *et al.*, 1995; Wang *et al.*, 1995; Schizophrenia linkage collaborative group for chromosomes 3, 6 and 8, 1996; Turecki *et al.*, 1997). Integrated physical maps of this region have been constructed to facilitate the identification of candidate genes for schizophrenia, and a large number of genes have been mapped (Davies *et al.*, 1995; Olavesen *et al.*, 1995; Olavesen *et al.*, 1997). Studies of the spinocerebellar ataxia type 1 (SCA1) gene, which has been mapped to chromosome 6p23, have failed to show a consistent association with schizophrenia (Wang *et al.*, 1996; Morris-Rosendahl *et al.*, 1997). As the chromosomal markers that yielded positive results span a relatively wide region on the short arm of chromosome 6, further study of this region is required in order to investigate alternative gene candidates. The gene for the transcription factor activator protein 2 (AP-2), may also be a possible candidate gene for schizophrenia. It has been mapped to a region near a marker D6S470, within chromosome 6p24 (Davies *et al.*, 1995; Olavesen *et al.*, 1997), that provided evidence of linkage to

schizophrenia (Schwab *et al.*, 1995; Straub *et al.*, 1995). AP-2 regulates the expression of genes required for the development of tissues of ectodermal origin. The ectoderm gives rise to the neural crest, brain, and skin tissues (Mitchell *et al.*, 1991; Williams and Tjian 1991a,b). AP-2 binds as a homodimer to the consensus recognition sequence GCCNNNGGC. This sequence is an important cis-regulatory element for a variety of genes, including proenkephalin, presenilin-2, the β_1 -adrenergic receptor, synapsin II, the α_3 nicotinic receptor subunit, the dopamine D1 receptor, dopamine β -hydroxylase, phenylethanolamine *N*-methyltransferase, metallothionein II_A, keratin and AP-2 (Imagawa *et al.*, 1987; Hyman *et al.*, 1989; Leask *et al.*, 1991; Minowa *et al.*, 1992; Bauer *et al.*, 1994; Petersohn *et al.*, 1995; Fornasari *et al.*, 1997; Ebert *et al.*, 1998; Kim *et al.*, 1998; Okawa *et al.*, 1998; Pennypacker *et al.*, 1998).

Most of the receptor subtypes for serotonin and dopamine stimulate or inhibit the formation of cyclic adenosine 3', 5'-monophosphate (cAMP) by coupling to adenylyl cyclase via the G protein subtypes (Martin and Humphrey, 1994; Missale *et al.*, 1998). Intracellular levels of cAMP are increased, and the effect of cAMP is then mediated by the activation of cAMP-dependent protein kinase A (PKA). PKA in turn regulates cellular function via phosphorylation of specific proteins (Della Fazia *et al.*, 1997; Sassone-Corsi, 1998). One of the best characterized PKA nuclear substrates is the transcription factor cAMP-responsive element (CRE)-binding protein (CREB). CREB stimulates transcription of genes that contain CREs (5'-TGACGTCA or similar motifs) in their promoter regions (Della Fazia *et al.*, 1997; Sassone-Corsi, 1998). Examples of these genes include somatostatin, the dopamine D1 receptor, the serotonin 2A receptor, the serotonin transporter, cholecystokinin, tyrosine hydroxylase, CREB, fos, proenkephalin, vasoactive intestinal peptide, synapsin I, and many others (Lee and Masson, 1993; Meyer *et al.*, 1993; Heils *et al.*, 1995; Zhu *et al.*, 1995; Minowa *et al.*,

1996; , Nielsen *et al.*, 1996; Montminy, 1997). Intriguingly, some of these genes, including the dopamine D1 receptor, the serotonin 2A receptor, the serotonin transporter, cholecystokinin, and synapsin I, are thought to be involved in schizophrenia. Evidence for this conclusion has come from studies showing alterations in the expression of these proteins in the central nervous system of schizophrenic patients (Virgo *et al.*, 1995; Burnet *et al.*, 1996; Bachus *et al.*, 1997; Hernandez and Sokolov, 1997; Okubo *et al.*, 1997; Tcherepanov and Sokolov, 1997). Taken together, the evidence suggests that the CREB gene is involved in the very same signal transduction and transcriptional regulation pathways that are thought to be involved in schizophrenia. It is therefore possible that the CREB gene is itself a candidate gene for the disease.

This study explored the hypothesis that modifications to the expression and/or function of the 5-HT_{1A} receptor, AP-2, or CREB, due to genetic mutations of these genes, may confer a predisposition towards schizophrenia by altering neural responsivity to environmental factors. The promoter region and the coding region of these genes were analyzed to identify genetic variants, and a case-control study was conducted to explore the association between polymorphisms in these genes and schizophrenia.

MATERIALS AND METHODS

DNA Samples

Written informed consent was obtained from each of the participants. The research protocol met the approval of the Ethics Committee of Tsukuba University. The patient group consisted of 87 unrelated schizophrenic patients, each of whom met the DSM-IV criteria for schizophrenia (American Psychiatric Association, 1994). Within this sample, 53 were male (mean age 46.3±12.7; mean age at onset 25.7±7.6) and 34 were female (mean age 47.3±16.3; mean age at onset 27.7±10.7). Sixty one of the 87 patients were

screened for the 5-HT_{1A} receptor gene, 87 patients for the AP-2 gene, and 80 of 87 patients for the CREB gene. Prior to the genomic DNA analysis, subtype and course specifiers were determined for each patient, using DSM-IV criteria. The controls consisted of 100 unrelated healthy volunteers (30 males, mean age 32.1±11.4; 70 females, mean age 43.1±12.0) who were hospital employees residing in the same area as the patient group. All of the patients and all of the control subjects were ethnically Japanese. Genomic DNA was prepared from whole blood samples collected in disodium EDTA (3mg/l). Genomic DNA was extracted using the sodium iodide method (DNA Extractor WB Kit; Wako Pure Chemical Industries, Tokyo).

Genotyping

1) Polymerase Chain Reaction (PCR) Conditions

Standard PCR was carried out in a 25 µl volume containing 100 ng genomic DNA, 8.25 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM of each dNTP, and 1U Taq DNA polymerase (Takara Biomedicals, Tokyo). After an initial 3 min denaturation phase at 94°C, 30 cycles were performed consisting of a denaturing step at 94°C for 30 sec, an annealing step at 55-71°C for 30 sec, and an extension step at 72°C for 30 sec; followed by a final extension phase at 72°C for 10 min, using a thermal cycler (Gene Amp 9600; Perkin-Elmer, Norwalk, CT).

[*Primers for the 5-HT_{1A} receptor gene*] The nucleotide sequence of the human 5-HT_{1A} receptor gene has been determined previously, and the DNA sequence numbering used herein for the 5-HT_{1A} receptor gene is based on previous reports (Kobilka *et al.*, 1987; Parks and Shenk, 1996). The 5-HT_{1A} receptor gene is intronless and consists of 1263 base pairs (bp) coding 421 amino acids and contains a TATA-less promoter. Six sets of PCR primers for the coding region (from nucleotide number 1 to 1307) and four sets of PCR primers for the promoter region (nucleotide number -855 to 45) were prepared

to produce overlapping fragments covering the complete coding region and the promoter region of the gene (Table 1). The 5'-terminus of each primer for the promoter region was labeled with indodicarbocyanine fluorescent dye (Pharmacia Biotech, Uppsala) to perform a fluorescence-based single-strand conformational polymorphism (SSCP) analysis.

[*Primers for the AP-2 gene*] The nucleotide sequence and the genomic structure of the human AP-2 gene have been reported previously (Bauer *et al.*, 1994). The nucleotide sequence numbering used herein for the AP-2 gene is based on this report. Mature AP-2 mRNA was spliced from 7 exons distributed over a 18 kb region of genomic DNA. An inhibitory AP-2 protein, designated as AP-2B, was generated by alternative usage after the fifth exon (Bauer *et al.*, 1994). The promoter of the AP-2 gene contains three AP-2 binding sites (nucleotides -622 to -614, -425 to -417, -385 to -378), an NF-1 binding site (nucleotides -365 to -353), an octamer binding site (nucleotides -339 to -330), a CT-rich repetitive element (nucleotides -240 to -103) (Bauer *et al.*, 1994; Creaser *et al.*, 1996), and a potential binding site for transcription factor NF-kB (nucleotides -1827 to -1818) (Baeuerle 1991). Fifteen sets of PCR primers were prepared to produce fragments covering each of these motifs, the CT-rich repetitive element in the promoter region, the entire coding region (including the AP-2B specific region), and all of the exon-intron boundaries of the AP-2 gene (Table 2). The 5'-terminus of each primer (not including the AP2STRPR primer) was labeled with indodicarbocyanine fluorescent dye (Pharmacia Biotech, Uppsala) to perform a fluorescence-based SSCP analysis. PCR was performed as described above, with the following minor modification: Dimethyl sulfoxide (final concentration, 5%) was added to the PCR reaction mixture for two primers sets; namely, the AP2PMF and AP2PMR primer set, and the AP2EX4PF and AP2EX4PR primer set.

[*Primers for the CREB gene*] The nucleotide sequence and the characterization of the promoter region of the human CREB gene have been

reported previously. Sequence numbering used herein for the CREB gene is based on a previous report (Meyer *et al.*, 1993). The promoter contained three CREs (nucleotide number -903 to -896, -119 to -112, -98 to -91) and three potential Sp1 binding sites (nucleotide number -299 to -284, -217 to -212, -150 to -145) (Meyer *et al.*, 1993). The potential binding site for the transcription factor NF- κ B (nucleotide number -499 to -490) (Baeuerle, 1991) was detected by inspection of the genomic sequence. Three sets of PCR primers were prepared to produce fragments covering each of these motifs (Table 3). The 5'-terminus of each primer was labeled with indodicarbocyanine fluorescent dye (Pharmacia Biotech, Uppsala) to perform a fluorescence-based SSCP analysis. PCR was carried out according to the methods described above, with the following modification: nested PCR (inner primers CREB-MF and CREB-MR) was performed using the outer primer pair; CREB-UF and CREB-DR.

2) Single-Strand Conformational Polymorphism (SSCP) Analysis

A DNA sequencer (ALF express; Pharmacia Biotech, Uppsala) equipped with a short gel plate was used to perform fluorescence-based SSCP analysis. One μ l of the PCR product was added to 14 μ l of the loading solution containing 99.5% deionized formamide and 0.5 % blue dextran. After denaturation at 96°C for 5 min, 2 μ l of the diluted mixture was applied to either a 10% polyacrylamide gel (PAG) (99:1, acrylamide: bisacrylamide ratio) or a 7% PAG (49:1, acrylamide: bisacrylamide ratio) containing 0.5 \times Tris-Borate-EDTA buffer (TBE) or 0.5 \times TBE buffer and 10% glycerol, respectively. The dimensions of the gel were 173 mm (height) \times 317 mm (width) \times 0.5 mm (thickness). The gels were subjected to electrophoresis at 20 W for 4~6 hr at 18°C. The data were analyzed using the software package Fragment Manager (Pharmacia Biotech, Uppsala).

As for the coding region of the 5-HT_{1A} receptor gene, alternatively SSCP analysis was performed as follows: 4 ul of the PCR product were mixed with 6 ul of the denaturing reaction solution (Perkin-Elmer), denatured for 10 min at 95°C, and cooled immediately on ice. The denatured products were loaded on a 10% PAG (99:1, acrylamide: bisacrylamide ratio) containing 0.5× TBE buffer, or 8~10% PAG (49:1, acrylamide: bisacrylamide ratio) containing 0.5× TBE buffer and 10% glycerol (140mm × 140mm × 1mm). Electrophoresis was performed at 7.1 V/cm for 12 hr at 10°C. Bands were visualized by ethidium bromide staining.

3) Sequencing of PCR Products

PCR products showing altered banding patterns in the SSCP analysis were purified by electrophoresis with a 1% agarose gel. Extraction was achieved by centrifugation using Microcon tubes (Amicon, Danver, MA). DNA sequences of the PCR products were directly determined from both directions using a cycle sequencing system (Dye Terminator Cycle Sequencing Ready Reaction and ABI PRISM 310 Genetic Analyzer; Perkin-Elmer).

4) Restriction Enzyme Assay

Msp I, *Rsa* I, *Fok* I, *Tfi* I, *Hae* III, *Nla* IV, *Mbo* II and *Hph* I were used to confirm mutations identified by the SSCP analysis and PCR direct sequencing, in accordance with the manufacture's recommendations. The digested products were visualized by electrophoresis in a 2~3 % agarose gel containing ethidium bromide.

Statistical Analysis

Significant differences in genotypic frequencies between patients and controls were analyzed using a χ^2 test (2 X 3 table) at a significance level of 0.05 (two-tailed). Differences in the allelic frequencies between patients and

controls were tested for significance using Fisher's exact test (2 X 2 table). Bonferroni correction for multiple comparisons was carried out to exclude type I errors. Linkage disequilibrium analysis was performed using the ASSOCIATE program (version 2.32) in conjunction with the LINKAGE UTILITY programs (Terwilliger and Ott 1994).

RESULTS

The 5-HT_{1A} Receptor Gene

A total of five variants (-51T→C, -152C→G, -321G→C, -480delA and -581C→A) in the promoter region of the 5-HT_{1A} receptor gene were identified using SSCP analysis and sequencing techniques (Table 4). Each site of the nucleotide substitutions was confirmed by restriction fragment-length polymorphism (RFLP) analysis using *Tfi* I (-51T→C), *Hae* III (-152C→G), *Nla* IV (-321G→C) and *Mbo* II (-581C→A) (Figure 1). There was no restriction enzyme commercially available to perform RFLP analysis of the -480delA variant. Figure 2 shows the results of PCR direct sequencing of the -480delA variant. In forward sequencing, a single base (A) deletion at nucleotide position -480 complementary to T was found (data not shown). Each of the variants (-51T→C, -152C→G, -321G→C and -581C→A) was detected in a different single control. The -480delA variant was found in only one patient. The patient was also heterozygous for the Gly272Asp variant. Using Fisher's exact test, no significant differences in genotypic and allelic frequencies of these variants were found between patients and controls.

Four variants (Pro16Leu, 294G→A, 549C→T and Gly272Asp) were identified in the coding region of the 5-HT_{1A} receptor gene (Table 4). The first variant was characterized by a single base pair substitution (47C→T) at the position of codon 16 and resulted in an amino acid exchange (Pro16Leu). The second was a silent mutation in codon 98 (Val) at a 294G→A substitution. The third was also a silent mutation in codon 183 (Pro) at a 549C→T substitution.

The fourth variant was characterized by a single base pair substitution (815G→A) at codon 272 and resulted in an amino acid exchange (Gly272Asp). Each site of the nucleotide substitutions was also confirmed by RFLP analysis using *Msp* I (Pro16Leu), *Rsa* I (294G→A) and *Fok* I (Gly272Asp). There was no restriction enzyme commercially available to perform RFLP analysis of the 549C→T mutation. SSCP analysis, PCR direct sequencing and RFLP analysis of the heterozygous genotype of the Gly272Asp mutation are presented in Figure 3.

Table 5 shows the frequencies of the variants in the coding region for patients and controls. Fisher's exact test was used to test for statistical significance. One homozygote and 5 heterozygotes of the Gly272Asp variant in the patient group, and 5 heterozygotes in the control group were identified. The allele frequencies for the patients and controls were 0.057 and 0.025, respectively. There were no significant differences in the genotypic and allelic frequencies found between the patient and control groups. The genotypic and allelic frequencies of the Pro16Leu, 294G→A and 549C→T variants were compared between patient and control groups. No significant differences were found. Furthermore, no evidence of linkage disequilibrium between the detected variants could be found.

The AP-2 Gene

Three variants in the promoter region of the AP-2 gene were identified by SSCP analysis and sequencing techniques (Table 6). The first variant, identified by a clearly altered SSCP banding pattern, was characterized by a single base pair substitution (G→C) at nucleotide position -90 by sequencing. The existence of the -90G→C nucleotide substitution was confirmed by RFLP analysis using *Msp* I. Figure 4 shows the SSCP analysis, sequencing, and RFLP analysis for the -90G→C variant. The second variant was characterized by a single base pair substitution (G→T) at nucleotide position -803 (Figure

5). There was no restriction enzyme commercially available to perform RFLP analysis of the 803G→T variant. The third variant was characterized by a single base pair substitution (G→A) at nucleotide position -1769. A single control sample was found to be heterozygotic for this mutation (Figure 6). However, there were no variants identified throughout the entire coding region of the AP-2 gene, or within its exon-intron boundaries.

Table 7 details the polymorphic status between the -90 and -803 loci in schizophrenics and control samples. The genotype of each sample at the loci was distinguished by clearly altered banding patterns in the SSCP analysis. The polymorphic status of each sample at each locus were almost identical. This suggests that a strong, but incomplete linkage disequilibrium exists between the -90G and -803G alleles, and between the -90C and -803T alleles.

Table 8 compares patients and controls for genotypic and allelic frequencies at the -90 and -803 loci of the AP-2 gene. Deviation from Hardy-Weinberg equilibrium was tested for each of the groups. Evidence of significant deviation was obtained in schizophrenics with continuous course ($\chi^2=4.05$, $df=1$, $P=0.044$). This will probably result from the relatively small population samples. However, no significant deviation was observed in other groups. No significant differences in genotypic and allelic frequencies at these loci were found between patients and controls. The limited size of the patient sample presented a problem for the statistical analysis. Because the frequency of the expected values smaller than 5 was more than 20%, a complete analysis of each of the individual subtypes and course specifiers within the patient group was not possible. For the purposes of the analysis, therefore the catatonic subtype and the undifferentiated subtype were grouped into a single category. The same was done for the patients in the single episode and other or unspecified pattern. Genotypic and allelic distributions were not found to be associated with any of the schizophrenic subtypes. However, when genetic data was analyzed with respect to the course specifiers, significant differences

were identified. A significant difference in the distribution of genotypes at the -90 locus was observed among patients with an episodic course, when compared against controls ($\chi^2=9.56$, $df=2$, $P=0.008$). A difference in the distribution of genotypes at the -803 locus was also observed among patients with an episodic course, relative to controls ($\chi^2=6.60$, $df=2$, $P=0.037$). However, the corrected P-values for the -90 and -803 loci were not significant, when the Bonferroni correction was made (the -90 locus, $P=0.064$; the -803 locus, $P=0.296$). No significant differences in allelic frequencies at the loci were found between the groups.

The CREB Gene

Two variants in the promoter region of the CREB gene were identified by SSCP analysis and sequencing techniques (Table 9). The first variant showing an altered banding pattern in the SSCP analysis was characterized using sequencing techniques as a single base pair substitution (T→C) at nucleotide position -933 (Figure 7). There was no restriction enzyme commercially available to perform RFLP analysis of the -933T→C variant. The second variant was characterized by a single base pair substitution (G→A) at nucleotide position -413. The -413G→A substitution creates a recognition site for the restriction enzyme *Hph* I. Therefore, the -413G→A variant site for the nucleotide substitution was confirmed by RFLP analysis using *Hph* I. Figure 8 shows the SSCP analysis, sequencing, and RFLP analysis for the -413G→A variant. Each of the variants was identified in a different single schizophrenic patient with an allele frequency of 0.6%. Both patients were heterozygotic for the variants. Neither of the variants could be detected in the control group. Using the Fisher's exact test, no significant differences in genotype and allele frequency were found between the patient and control groups (Table 9).

Table 10 shows the clinical characteristics of the schizophrenic patients possessing genetic variants identified in the present study. Although the age of

onset was comparatively early (15 years of age) in the male patient possessing the -413G→A variant, there was otherwise nothing particularly unusual about this patient. In contrast, the female patient with the -933T→C variant had a family history of schizophrenia (an uncle) (Figure 9), and displayed the earliest age of onset for the entire patient sample (13 years of age). Moreover, this patient displayed some unusual clinical characteristics. Electroencephalography (EEG) revealed abnormalities in the slow-wave, while CT, MRI and neurological examination were within normal limits (Figure 10). The patient also experienced visual hallucinations, conversion symptoms and intellectual impairment. The patient's IQ was 54, as assessed by the WAIS-R (Wechsler Adult Intelligence Scale-Revised) test. The patient's other symptoms, including auditory hallucinations and persecutory delusions, were more typical of the disease. The WAIS-R analysis suggested that her intellectual impairment may have resulted from reduced capacity to retain long-term memory (Figure 11).

DISCUSSION

A missense mutation (815G→A) of the human 5-HT_{1A} receptor gene, leading to an amino acid exchange (Gly272Asp) reported here is apparently the novel naturally occurring variant. The 5-HT_{1A} receptor belongs to the family of the G protein-coupled receptors. The receptors generally contain seven hydrophobic transmembrane helices, which include three extracellular and three intracellular loops (Varrault *et al.*, 1994). The Gly272Asp mutation was located in the intermediate portion of the third intracellular loop (Kobilka *et al.*, 1987). Mutagenesis studies have shown that the third intracellular loop is implicated in G-protein binding and activation. The peptides from the entire second intracellular loop (amino acid 133-153) as well as the C-terminal part of the third intracellular loop (amino acid 330-346) can activate G-proteins, and function to regulate the receptor effector (Varrault *et al.*, 1994). The amino acid residue (272Gly) is conserved between human and rat 5-HT_{1A}

receptors (Albert *et al.*, 1990), indicating a possible conservation of function. This lends further support to the notion that the Gly272Asp substitution may alter the receptor's signal transduction function, through an alteration in G-protein coupling. Three further variants (Pro16Leu, 294G→A and 549C→T) were identified in the coding region of the 5-HT_{1A} receptor gene. Each of these variants has been reported previously (Erdmann *et al.*, 1995; Harada *et al.*, 1996; Xie *et al.*, 1995). In the previous studies, the allelic frequencies of the Pro16Leu and 294G→A variants, which were identified in the Japanese population, were reported to be 2.29% (Harada *et al.*, 1996) and 3.48% (Inayama *et al.*, 1996). These values are close to those found in the present study (3.42% and 3.42%, respectively). The allele frequency of the 549C→T mutation, which had been reported in subjects of German origin to be 0.94% (Erdmann *et al.*, 1995) is lower than that observed in the present study (2.80%). No significant differences in allelic and genotypic frequencies of these variants were found between patients and controls. The present study also confirms a previous report suggesting that there is no association between the 294G→A mutation and schizophrenia (Inayama *et al.*, 1996). Note however, that variants in the coding region of the 5-HT_{1A} receptor gene may prove to be a fruitful avenue of research for an association study of other neuropsychiatric disorders such as depression, anxiety disorders, obsessive-compulsive disorder, eating disorders, seasonal affective disorder, alcoholism, Alzheimer's disease, autism, attention deficit disorder, Tourette's syndrome and sociopathy, in which dysfunction in serotonergic function has been implicated (Dubovsky, 1994).

The entire coding region of the AP-2 gene, containing the AP-2B specific region, and all of the exon-intron boundaries, were analyzed in order to identify genetic variations that could lead to altered protein function. However, no variant was detected in these regions. This suggests that there may be strong conservation of the coding region of the AP-2 gene. It may also suggest that a

severe phenotype might arise from alteration or loss of protein function due to mutations of the AP-2 gene. The latter conclusion finds support from studies of AP-2 null mice, which display profound developmental abnormalities (Schorle *et al.*, 1996; Zhang *et al.*, 1996).

In total, five rare nucleotide sequence variants (-51T→C, -152C→G, -321G→C, -480delA and -581C→A) were identified in the 5-HT_{1A} receptor gene. Three variants were identified in the AP-2 gene, of which the first two were relatively common (-90G→C, -803G→T) and the third one was rare (-1769G→A). Finally, two rare variants (-933T→C, -413G→A) were identified in the CREB gene. All of the variants appeared in the promoter region of their respective genes. Polymorphic status at both loci in the AP-2 gene suggested that there was strong linkage disequilibrium between the -90 and -803 loci. The present study may be the first report of nucleotide sequence variants in the promoter region of the genes encoding the 5-HT_{1A} receptor, AP-2, and CREB. However, none of the variants reported in this study corresponded to the motifs relating to transcriptional regulation. This suggests that the variants are not likely to have any functional influence on the transcriptional activity of these genes. Furthermore, our data provide no evidence of an association between schizophrenia and the variants in the promoter region of the 5-HT_{1A}, AP-2, and CREB genes.

Caution must be exercised in interpreting the negative results revealed in the association analysis. Firstly, the comparatively small sample size may have given rise to type II statistical errors. A substantially larger sample may yet yield significant results. Secondly, the case-control experimental method carries with it the possibility of a false negative result due to a genetic bias in the particular sample groups included for analysis. Note however that the effects of population stratification are reduced by the fact that controls and schizophrenic patients in this study were sampled from a more genetically homogenous population group.

Significant differences in the distribution of genotypes at the -90 and -803 loci in the AP-2 gene were observed in patients with an episodic course, compared to controls, although no significant differences in allelic frequencies were observed between the groups. However, the corrected P-values for the -90 and -803 loci were not significant after Bonferroni correction for multiple comparisons. This suggests that the significant associations between the polymorphisms (-90G→C and -803G→T) and the episodic course of schizophrenia are likely to be type I statistical error, although application of the Bonferroni correction is very conservative because many of the comparisons may not be strictly independent. On the other hand, each set of patients subdivided using DSM-IV became quite small. This may make any conclusion preliminary. A larger sample size is required to assess the association between the AP-2 gene polymorphisms and the diagnostic subdivisions of schizophrenia.

Considering the genetic heterogeneity of predisposition towards schizophrenia, it should be noted that the variants in the CREB gene were at least confined to the patient group, although these variants were rare. The possibility that these variants confer a genetic predisposition towards minor subgroups of schizophrenia cannot be eliminated. Indeed, the patient with the -933T→C variant displayed some unusual clinical characteristics in addition to more typical schizophrenic symptoms. This possibility is given further support when one considers the intellectual impairments that were observed in this patient. Mice with a targeted mutation of the CREB gene display profound deficiencies in long-term memory function, but do not display any other overt abnormalities (Bourtchuladze *et al.*, 1994).

Recent studies have suggested that functional polymorphisms in the promoter region of genes expressed in the brain may be involved in the susceptibility to neuropsychiatric disorders, including schizophrenia, affective disorder, panic disorder, and Alzheimer's disease (Collier *et al.*, 1996; Arinami

et al., 1997; Artiga *et al.*, 1998; Ohara *et al.*, 1998; Wang *et al.*, 1998). Many genes contain AP-2 or CREB binding sites. These genes are apparently involved in many different physiological functions. Several studies have suggested that dysfunction involving either AP-2 or CREB lead to severe phenotypes, including the developmental abnormalities observed in AP-2 null mice (Schorle *et al.*, 1996; Zhang *et al.*, 1996) or Rubinstein-Taybi syndrome in the CBP (Petrij *et al.*, 1995). However, the variants located in the promoter region of the AP-2 or CREB genes would be expected to confer a more subtle effect on their protein expression to the extent that severe phenotypes does not arise. These effects could be involved in the development of particular schizophrenic phenotypes.

The polymorphism in the promoter region of the transcription factor is of particular interest. The results suggested that there was an association between the episodic schizophrenic phenotype and this polymorphism. Note that episodic schizophrenia demonstrates the plastic nature of disease. Polymorphism in the promoter region of the transcription factor, which may play a central role in transsynaptic regulation of neural gene expression, could modify long-term and plastic effects of environmental factors on both the developing and adult brain (Comb *et al.*, 1987; Hyman and Nestler, 1993).

In order to screen the variants of the genes coding 5-HT_{1A}, AP-2, and CREB we used an improved SSCP analysis method (Makino *et al.*, 1992; Xie *et al.*, 1997). However, the possibility will always remain that the SSCP analysis will fail to detect a genetic variant, as the technique is not 100% sensitive (Hayashi 1992; Jordanova *et al.*, 1997).

This study provides preliminary evidence that schizophrenia characterized by an episodic course may correlate to the -90G→C and -803G→T polymorphisms in the promoter region of the AP-2 gene. In addition, the results suggest that the -933T→C variant may confer additional and uncharacteristic clinical features of the disease. However, a larger sample size

will be necessary to determine whether the AP-2 and CREB gene confer susceptibility towards these schizophrenic phenotypes at a statistically significant level. Polymorphic sites identified in the present study did not correspond with any of the published putative consensus sequences of transcriptional factors. However, we can not exclude the possibility that these polymorphic sites could be involved in the binding cis-elements of unknown transcriptional factors. Further detailed analysis is necessary to determine whether the transversions from -90G to C and from -803G to T in the AP-2 gene, and the transition from -933T to C in the CREB gene effect transcriptional activity of these genes. Finally, analysis of the coding region of the CREB gene will be necessary.