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Synonymous mutations in the human *dopamine receptor D2 (DRD2)* affect mRNA stability and synthesis of the receptor

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Although changes in nucleotide sequence affecting the composition and the structure of proteins are well known, functional changes resulting from nucleotide substitutions cannot always be inferred from simple analysis of DNA sequence. Because a strong synonymous codon usage bias in the human *DRD2* gene, suggesting selection on synonymous positions, was revealed by the relative independence of the G + C content of the third codon positions from the isochoric G + C frequencies, we chose to investigate functional effects of the six known naturally occurring synonymous changes (C132T, G423A, T765C, C939T, C957T, and G1101A) in the human *DRD2*. We report here that some synonymous mutations in the human *DRD2* have functional effects and suggest a novel genetic mechanism. 957T, rather than being 'silent', altered the predicted mRNA folding, led to a decrease in mRNA stability and translation, and dramatically changed dopamine-induced up-regulation of *DRD2* expression. 1101A did not show an effect by itself but annulled the above effects of 957T in the compound clone 957T/1101A, demonstrating that combinations of synonymous mutations can have functional consequences drastically different from those of each isolated mutation. C957T was found to be in linkage disequilibrium in a European-American population with the -141C Ins/Del and *Taq*I 'A' variants, which have been reported to be associated with schizophrenia and alcoholism, respectively. These results call into question some assumptions made about synonymous variation in molecular population genetics and gene-mapping studies of diseases with complex inheritance, and indicate that synonymous variation can have effects of potential pathophysiological and pharmacogenetic importance.

INTRODUCTION

In coding regions of genes, single nucleotide polymorphisms (SNPs) are categorized as either synonymous (also called 'silent') for those that do not change amino acid sequence or missense for those that do. In human genes, roughly 99.8% of DNA sequence variations do not alter the primary sequences of proteins (1), and synonymous SNPs show a higher frequency than both missense SNPs and the SNPs in the non-coding regions (1,2). Synonymous SNPs are the most polymorphic, indicating that most such mutations may be functionally neutral. However, this does not mean that every synonymous site is non-functional or neutral. Studies in *E.coli*, yeast, and *Drosophila* support translation selection for major codons, and there is a strong correlation between bias in synonymous codon (SC) usage and the gene expression level (3). In the human genome, GC-ending codons are significantly more frequent in constitutive than in alternative exons, suggesting there may also be a translation selection for synonymous codon usage (4). An effect of synonymous codon usage on gene expression is supported by the detection of epistatic interactions between nucleotides that are important in maintaining

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pre-mRNA/mRNA secondary structures in the *Drosophila alcohol dehydrogenase* genes (*Adh* and *Adhr*) (5–7). A molecular evolutionary analysis of *BRCA1* also provides supportive evidence of selection for RNA or DNA structure acting on synonymous codon usage (8). In addition, some synonymous changes in humans have been shown to cause genetic disorders by exon skipping (9,10). Since it is commonly believed that association studies with candidate genes should be preferentially conducted with functional SNPs and other functional mutations, the identification of nucleotide substitutions associated with functional changes should have important implications for the design and interpretation of disease association studies.

G + C content of the third positions of codons (GC3) has been classically used to investigate whether natural selection influences synonymous codon usage (3–5,11–13). However, the isochoric structure of the human genome, in relation to the distribution of G + C content across the genome, might confound the assessment of the influence of selection on synonymous positions. This is because the base composition of coding sequences tends to reflect the variation in G + C content along chromosomes (11,14,15). Therefore, we investigated the departure between the GC3 of a gene and the G + C frequency observed in adjacent non-coding regions which can be used as a measure of gene specific forces (e.g. selection) on synonymous mutations beyond isochoric mutational tendencies.

We studied those genes showing the strongest departure from isochoric G + C content as the best candidates for the detection of functional changes resulting from synonymous mutations. In particular, we focused on the study of human G protein-coupled receptor (GPCR) genes, many of which are implicated in psychiatric disorders. Our analysis showed that among 35 closely related human GPCR genes (five dopamine receptors, 12 serotonin receptors, nine adrenergic receptors, five cholinergic receptors, and four histamine receptors), the GC3 in *DRD2* departed most clearly from its neighboring isochoric G + C content (Fig. 1). The results of our analysis suggested that selection maintains the GC3 of *DRD2* at a level well above the G + C content of the isochore in which the gene resides, making *DRD2* an ideal candidate to investigate the possible functional consequences of synonymous SNPs.

Dopamine receptors are involved in motor control, neuroendocrine function, reward and reinforcement, and cognition, and are targets in the pharmacologic therapy of schizophrenia, Parkinson's disease, Tourette's syndrome, tardive dyskinesia, and Huntington's disease (16). Furthermore, due to its central role, *DRD2* is among the most tested candidate genes in psychiatric disorders. Multiple polymorphisms as well as alternative splicing have been described for *DRD2* (17–25). *DRD2* has two major isoforms (designated as long and short) generated by alternative splicing of exon 6 (encoding 29 amino acids) from the mRNA (17). Both isoforms display distinct functions *in vivo* (24) as well as a differential ability to increase the steady state receptor concentration induced by dopamine in *DRD2*-transfected CHO-K1 cells (26). Four infrequent missense mutations (V96A, V154I, P310S and S311C) (19,21) and a polymorphism (-141C Ins/Del) in the promoter region (27) of *DRD2* have been described. Some of the missense SNPs and -141C Ins/Del have been reported to be associated with schizophrenia, although replication of these finding has yielded inconsistent results (22,23). Six known *DRD2* synonymous polymorphisms (C132T, G423A, T765C, C939T, C957T, and G1101A) (17,18,20,21) had been presumed to be functionally silent and therefore have not been investigated for a relationship with disease. In this study, however, we have examined the functional properties of the synonymous changes in *DRD2*, and have found evidence that two of the six synonymous SNPs have marked functional consequences on *DRD2* mRNA stability and dopamine-regulated *DRD2* expression. We also suggest a novel mechanism by which these two functional synonymous mutations in *DRD2* can affect its expression and regulation.

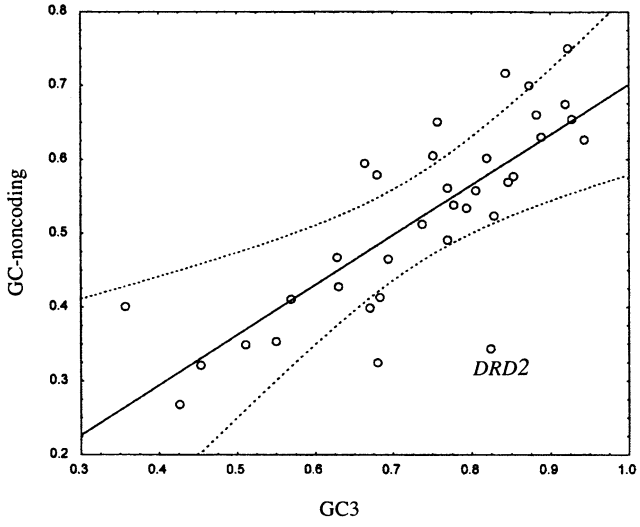

Figure 1. Relationship between GC3 of 35 human GPCR genes and G + C content in adjacent non-coding (GC non-coding) sequences. Continuous and dashed lines indicate the linear regression and the 99.9% confidence intervals, respectively. Note that the GC3 of *DRD2* is significantly higher than its GC non-coding ($G = 216.3$, $p < 1 \times 10^{-6}$, G test for goodness of fit). GC3 was estimated from the consensus human cDNA sequences for the 35 GPCRs, and GC-noncoding was estimated using an average size of 3.5 kb.

RESULTS

Codon-usage bias in *DRD2*

We first analyzed the departure between the GC3 of a gene and the G + C frequency observed in adjacent non-coding regions as a measure of the isochoric G + C content. We studied a total of 35 human GPCR genes, including dopamine receptors ($n = 5$), serotonin receptors ($n = 12$), cholinergic receptors ($n = 5$), adrenergic receptors ($n = 9$), and histamine receptors ($n = 4$). As expected, there is a strong correlation (Spearman's $r = 0.775$, $P < 1 \times 10^{-6}$) between GC3 and GC non-coding (isochoric G + C content) (Fig. 1). However, GC3 in *DRD2* departs strongly from the general trend, showing a significant relative excess of GC3 ($G = 216.03$, $P < 1 \times 10^{-6}$). It could be argued that such a skewed GC3/GC non-coding might manifest a selective pressure to maintain or diminish the GC content of noncoding sequences that immediately flank a gene and two genes with the same GC3 could show a markedly

different GC3/GC non-coding relationship based upon the characteristics (and base compositions) of their promoters. To address this possibility, and assuming that the isochoric environment would affect equally all genes located in the same genomic region, we have performed additional analyses of *DRD2* and genes immediately located at its 5′ and 3′ end based on the chromosome 11 reference genomic contig (NT_035088.1). The results of these analyses show that GC3 in *DRD2* is higher than in genes flanking *DRD2* while the GC-noncoding of these neighboring genes and *DRD2* is not different. Specifically, GC3 in *DRD2* is greater ($P < 1 \times 10^{-6}$) than that in genes immediately flanking it on both sides, using two ($G = 66.7$), four ($G = 84.6$) or six ($G = 142.4$) flanking genes. At the same time, the GC non-coding immediately 5′ (1 kb) to *DRD2* does not show any reduction compared with that in genes immediately flanking it using either two ($G = 1.55$, $P > 0.2$), four ($G = 1.29$, $P > 0.25$) or six ($G = 0.383$, $P > 0.5$) flanking genes (both 5′ and 3′ relative to *DRD2*), and equivalent results are obtained using a larger 5′ sequence (e.g. 3 kb) and also after removing from the analysis the 300 bp immediately 5′ to the start codon reflecting the mean size of human 5′- UTRs (28). These analyses indicate that the high GC3 in *DRD2* relative to the isochoric G + C is restricted to this gene and that there is no particular reduction of GC non-coding immediately flanking *DRD2*. Altogether, these results strongly suggest that a selective effect on third codon positions of *DRD2* is present.

An independent line of evidence supporting the hypothesis that bases at the third codon positions of *DRD2* are under selection can been seen upon examination of data in Table 1. Table 1 shows the SC usage for *DRD2*, 35 GPCRs, and 11 ribosomal proteins, and the average SC usage for the human genome. We chose ribosomal proteins as a comparison group since they are highly expressed and thus potentially under selection for high codon usage bias. As can be seen from the lowest and highest 5% of codon usage for all the human genes in Table 1, the variation of codon usage is rather high, and this variation is from multiple sources including the isochore environment. We therefore have chosen to focus upon the portion of data from Table 1 that allows separation of the forces behind isochoric G + C variation (which is by nature independent of gene orientation, i.e. it is not 'strand specific') from other influences. The forces behind isochoric G + C variation are expected to affect the G and C content concurrently, but they could not explain a different behavior for G- and C-ending synonymous codons. Hence, an independent line of evidence from the previous analyses (Fig. 1) that the bases at the third codon positions of *DRD2* are under selection is provided by the study of G-ending and the C-ending synonymous codons in 4-fold-degenerate (chosen to have the opportunity to show, if present, independent variation of G- and C-ending codons) SC families (e.g. GUC and GUG codons of the valine family). Note that if the isochoric G + C variation was responsible for the increased GC3 in *DRD2*, G- and C-ending synonymous codons in 4-fold-degenerate SC families would be expected to be similarly influenced. Table 1 shows an independent behavior of G-ending and C-ending synonymous codons in *DRD2* from each other when compared to the global human SC usage. This observation suggests that selection is acting on synonymous sites not only to maintain a high GC3 content but probably also on other levels. Because of these preliminary findings, we next examined the functional consequences of synonymous mutations naturally segregating at *DRD2* (17,18,20,21).

Decreased *in vitro* translation efficiency for mRNA carrying 957T

We first investigated the *in vitro* translation of *DRD2* mRNAs carrying various synonymous mutations. We compared protein synthesis at each time point by presenting the data as percentage of the amount of protein synthesis by wild type (WT) at 60 min. The *DRD2* mRNA carrying the 957T mutation showed a 50% decrease in the protein synthesis level as an indication of decreased translation compared with the others at 60 min (Fig. 2A). When expressed alone, the 1101A mutation did not affect the translation activity. Interestingly, the mRNA carrying the combined mutations 957T/1101A exhibited a protein synthesis pattern similar to WT *DRD2* mRNA, suggesting that the decreased translation associated with the 957T mutation was annulled in the presence of 1101A (Fig. 2A).

Mutant mRNA with the 957T mutation is less stable

Since the C957T mutation and another four of the studied mutations (C132T, G423A, C939T, and G1101A) are all changes from a preferred codon to a non-preferred one, we inferred that the decrease in protein synthesis associated with 957T is unlikely to be a general effect of codon preference. Therefore, we next examined the possible effect of synonymous SNPs on the mRNA stability. Stably transfected CHO (Chinese hamster ovary)-K1 cells were treated with actinomycin D (ActD) to inhibit transcription. *DRD2* mRNA and the β-*actin* (*ACTB*) control mRNA showed a similar decay in all clones except for 957T (Fig. 2C), a difference that is detectable with (Fig. 2B) or without normalization by *ACTB* mRNA (data not shown). *DRD2* mRNA carrying the 957T mutation decayed significantly faster than WT mRNA, and the mRNA half-life was decreased from approximately 8 h (WT) to 4 h (957T) (Fig. 2B). The other synonymous mutations (132T, 423A, 765C, 939T and 1101A) did not show any effect on mRNA stability (Fig. 2B). While 1101A did not have an effect by itself, it again annulled the effect of 957T in the compound clone (Fig. 2B).

Effect of 957T on dopamine induced up-regulation of *DRD2* expression

Work by others has shown that dopamine up-regulates mRNA of the *DRD2* long isoform 2-fold in CHO cells transfected with *DRD2* cDNA through an unknown mechanism (26). Given the strong selection for synonymous codon usage in *DRD2* and the demonstrated function of synonymous mutations 957T and 1101A, we hypothesized that 957T and possibly other synonymous mutations might alter this dopamine-mediated effect, although most synonymous mutations are not expected to be functional. We then examined the effect of synonymous mutations on dopamine-mediated changes in *DRD2* mRNA expression. Transfected cells were treated with dopamine, and *DRD2* mRNA levels in cultured cells were measured at various

Table 1. SC usage in *DRD2*; all dopamine, serotonin, adrenergic, cholinergic, and histamine GPCRs; and 11 ribosomal proteins vs. the human genome[a]

codon	*DRD2*	DRDs, HTRs, ADRs, CHRs, & HRs	ribosomal proteins	*Homo sapiens* 5th percentile	*Homo sapiens* average	*Homo sapiens* 95th percentile	codon	*DRD2*	DRDs, HTRs, ADRs, CHRs, & HRs	ribosomal proteins	*Homo sapiens* 5th percentile	*Homo sapiens* average	*Homo sapiens* 95th percentile	codon	*DRD2*	DRDs, HTRs, ADRs, CHRs, & HRs	ribosomal proteins	*Homo sapiens* 5th percentile	*Homo sapiens* average	*Homo sapiens* 95th percentile	codon	*DRD2*	DRDs, HTRs, ADRs, CHRs, & HRs	ribosomal proteins	*Homo sapiens* 5th percentile	*Homo sapiens* average	*Homo sapiens* 95th percentile
UUU	0.05	0.30	0.34	0.00	0.45	0.82	UCU	0.07	0.21	0.35	0.00	0.30	0.56	UAU	0.18	0.27	0.33	0.00	0.43	0.85	UGU	0.33	0.29	0.29	0.00	0.45	1.00
UUC	0.95	0.70	0.66	0.13	0.55	1.00	UCC	0.87	0.47	0.49	0.06	0.36	0.68	UAC	0.82	0.73	0.67	0.00	0.57	1.00	UGC	0.67	0.71	0.71	0.00	0.55	1.00
UUA	0.00	0.30	0.19	0.00	0.37	0.71	UCA	0.07	0.15	0.07	0.00	0.24	0.50	UAA	x	x	x	x	x	x	UGA	x	x	x	x	x	x
UUG	1.00	0.70	0.81	0.00	0.63	1.00	UCG	0.00	0.17	0.09	0.00	0.09	0.33	UAG	x	x	x	x	x	x	UGG	1.00	1.00	1.00	1.00	1.00	1.00
CUU	0.00	0.09	0.16	0.00	0.16	0.44	CCU	0.04	0.18	0.34	0.00	0.28	0.53	CAU	0.14	0.30	0.30	0.00	0.41	0.86	CGU	0.06	0.08	0.19	0.00	0.14	0.50
CUC	0.42	0.31	0.23	0.05	0.24	0.43	CCC	0.59	0.44	0.29	0.00	0.33	0.61	CAC	0.86	0.70	0.70	0.00	0.59	1.00	CGC	0.44	0.52	0.40	0.00	0.32	0.64
CUA	0.02	0.06	0.06	0.00	0.09	0.26	CCA	0.26	0.18	0.22	0.00	0.27	0.54	CAA	0.00	0.21	0.17	0.00	0.25	0.60	CGA	0.17	0.11	0.13	0.00	0.19	0.56
CUG	0.56	0.54	0.55	0.19	0.51	0.75	CCG	0.11	0.20	0.15	0.00	0.11	0.33	CAG	1.00	0.79	0.83	0.38	0.75	1.00	CGG	0.33	0.29	0.28	0.00	0.35	0.67
AUU	0.21	0.24	0.34	0.00	0.36	0.64	ACU	0.17	0.16	0.28	0.00	0.24	0.50	AAU	0.26	0.33	0.35	0.00	0.46	0.80	AGU	0.06	0.21	0.36	0.00	0.38	0.80
AUC	0.76	0.68	0.63	0.09	0.49	1.00	ACC	0.59	0.47	0.46	0.07	0.36	0.67	AAC	0.74	0.67	0.65	0.14	0.54	1.00	AGC	0.94	0.79	0.64	0.14	0.62	1.00
AUA	0.03	0.08	0.04	0.00	0.16	0.40	ACA	0.14	0.18	0.16	0.00	0.28	0.53	AAA	0.30	0.28	0.25	0.00	0.42	0.72	AGA	0.27	0.33	0.53	0.00	0.50	0.94
AUG	1.00	1.00	1.00	1.00	1.00	1.00	ACG	0.10	0.18	0.09	0.00	0.12	0.33	AAG	0.70	0.72	0.75	0.26	0.58	1.00	AGG	0.73	0.67	0.47	0.00	0.50	1.00
GUU	0.08	0.09	0.13	0.00	0.18	0.43	GCU	0.21	0.17	0.36	0.05	0.26	0.50	GAU	0.25	0.29	0.45	0.00	0.46	0.80	GGU	0.17	0.12	0.24	0.00	0.16	0.36
GUC	0.56	0.33	0.32	0.00	0.24	0.46	GCC	0.62	0.52	0.42	0.12	0.40	0.67	GAC	0.75	0.71	0.55	0.18	0.54	0.95	GGC	0.42	0.51	0.40	0.06	0.34	0.64
GUA	0.03	0.06	0.11	0.00	0.11	0.33	GCA	0.07	0.12	0.17	0.00	0.23	0.48	GAA	0.00	0.28	0.33	0.03	0.42	0.77	GGA	0.08	0.11	0.18	0.00	0.25	0.53
GUG	0.33	0.52	0.45	0.17	0.47	0.76	GCG	0.10	0.19	0.06	0.00	0.11	0.31	GAG	1.00	0.72	0.67	0.22	0.58	0.96	GGG	0.33	0.26	0.18	0.00	0.25	0.45

[a] The codon usage frequencies in SC families for the long isoform of the human *DRD2* (444 total codons; NM_000795.2), the 5 dopamine, 12 serotonin, 9 adrenergic, 5 cholinergic, and 4 histamine GPCRs (15,651 total codons; NM_000794.2, NM_000795.2, NM_000796.2, NM_000797.1, NM_000798.2, NM_000524.1, NM_000863.1, NM_000864.1, NM_000865.1, NM_000866.1, NM_000621.1, NM_000867.1, NM_000868.1, NM_000870.1, NM_024012.1, NM_000871.1, NM_000872.2, NM_000680.1, NM_000679.2, NM_000678.2, NM_000681.2, NM_000682.2, NM_000683.2, NM_000684.1, NM_000024.3, NM_000025.1, NM_000738.1, NM_000739.1, NM_000740.1, NM_000741.1, NM_012125.1, NM_000861.2, NM_022304.1, NM_007232.1, NM_021624.2), 11 highly expressed genes for human ribosomal proteins (2,223 total codons; NM_001013.2, NM_005617.2, NM_000990.2, NM_000967.2, NM_000968.2, NM_001020.2, NM_001015.2, NM_000977.2, NM_001022.2, NM_001003.2, NM_001004.2), and 41,507 human coding DNA sequences (CDS) (18,611,700 total codons; accessed 04/10/02 from the Codon Usage Database, ftp://ftp.kazusa.or.jp/pub/codon/current/species/Homo_sapiens.pri) with the 5th and 95th percentiles flanking the average usage for the 41,507 human CDS. Each sixfold-degenerate SC family was split into a twofold and fourfold "subfamily" to reflect the subfamilies' low probability of mutational contact.

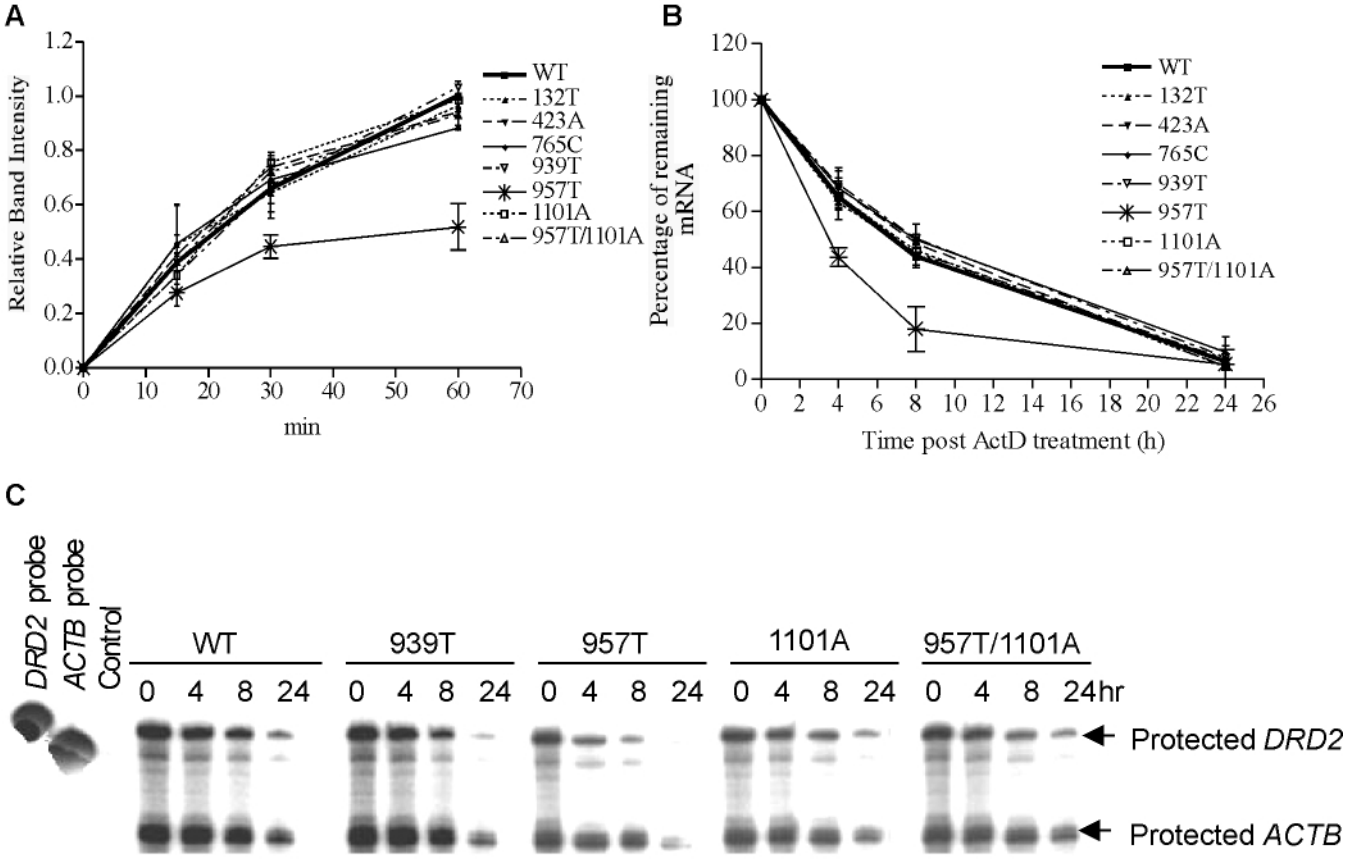

Figure 2. *In vitro* translation and stability of *DRD2* mRNAs carrying various synonymous mutations. (**A**) Time course changes in the *in vitro* protein synthesis of the receptor. Data are presented as proportions of the amount of WT at 60 min. Only the mutant 957T is significantly different from WT ($P < 0.001$, by two-way ANOVA). Each data point represents the mean relative band intensity from two to three independent experiments. (**B**) Time course changes in the remaining amount of *DRD2* mRNA after ActD treatment. The data plotted are from at least three independent experiments. All the data were normalized by *ACTB* expression. Only the 957T *DRD2* mRNA showed a significant difference from WT ($P = 0.0014$, by two-way ANOVA). (**C**) Representative autoradiograph of RPA. Cell lysate used in RPA was from 5×10^5 cells at the indicated times (0, 4, 8 and 24 h) after treatment with ActD. The control showed the RNase digestion pattern of probes in a reaction without cell lysate.

time points. Dopamine up-regulated the cellular *DRD2* mRNA level with the peak increase at 4 h [1.5–1.8-fold, $P < 0.0001$, by two-way ANOVA (analysis of variance)] (Fig. 3A). However, 957T consistently showed a decreased response compared with WT. The dopamine-induced up-regulation of *DRD2* mRNA could be blocked by (−)-sulpiride (data not shown), indicating that the effect of dopamine here is specifically mediated by *DRD2*. Clone 957T/1101A did not show a significantly diminished response, once more suggesting a compensatory effect of 1101A. These changes are likely to result in changes of receptor synthesis.

We next addressed whether the effects of 957T on dopamine-induced up-regulation of *DRD2* mRNA could alter *DRD2* expression in cell membrane preparations. Stably transfected CHO cells expressing either WT or 957T *DRD2* constructs were used to quantitate [^{3}H]methylspiperone-specific binding sites determined by saturation binding studies. The density of specific [^{3}H]methylspiperone binding sites was similar between the untreated *DRD2* WT transfected cells (47.5 ± 10.4 pmol/mg of membrane protein) and 957T (36.7 ± 5.4 pmol/mg). Treatment with dopamine resulted in a significant ($P < 0.05$ compared with baseline by two-tailed *t*-test) increase in [^{3}H]methylspiperone-specific binding as determined by single-point measurements of radioligand binding after 6 h of exposure to drug in both WT (107.9 ± 16.8 pmol/mg) and 957T receptor expressing cells (80.1 ± 8.2 pmol/mg). This increase was sustained after 24 h of treatment with dopamine in both WT (118.0 ± 6.6 pmol/mg) and 957T receptor expressing cells (90.5 ± 12.9 pmol/mg). A significant difference of receptor expression between WT and 957T clones appeared after 6 h ($P = 0.0076$, *t*-test) and 24 h ($P = 0.0054$, *t*-test) of treatment with dopamine. These results indicated that 957T decreases the dopamine-induced up-regulation of receptor expression at both the mRNA level and the membrane protein level.

Dopamine differentially stabilized WT and 957T *DRD2* mRNA

The effect of 957T on dopamine-induced up-regulation of *DRD2* mRNA in transfected CHO cells could be explained by an effect of on transcription of *DRD2* mRNA or by a differential stability of *DRD2* mRNA, i.e. a post-transcriptional event. We therefore studied *DRD2* mRNA turnover after blocking transcription by treating cells with ActD. We found that dopamine treatment

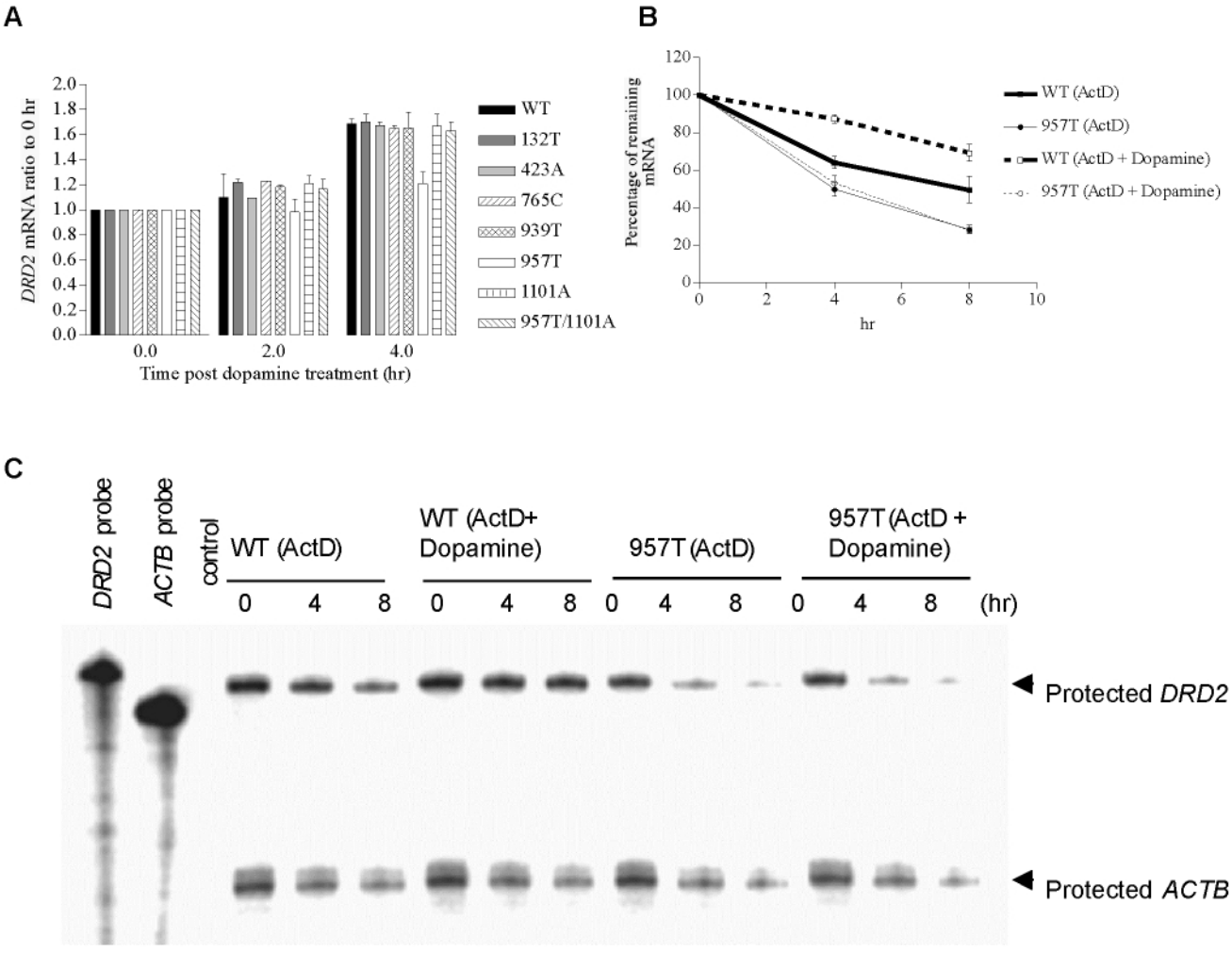

Figure 3. Dopamine induced up-regulation of *DRD2* mRNA in transfected CHO cells and differential effects of dopamine on the stability of WT and 957T *DRD2* mRNA. (**A**) Time course changes of *DRD2* mRNA level upon dopamine treatment. Data are presented as a ratio calculated by dividing the *DRD2* mRNA level at the indicated time point by the mRNA level at 0 h of dopamine treatment. Only 957T cells had a significant difference from WT ($P = 0.0273$, by two-way ANOVA). (**B**) Time course changes of *DRD2* mRNA level in the presence of ActD. Dopamine only significantly changed the mRNA stability of WT ($P < 0.001$, by ANOVA). For both (A) and (B), data at each time point were from four independent experiments and were normalized by *ACTB* expression (assuming the same *ACTB* decaying pattern in all the cell clones, and the acquired *DRD2* data were adjusted accordingly). (**C**) Representative autoradiograph of an RPA in (B). Dopamine was applied or not to stable-transfected CHO cells in the presence of ActD. Cell lysate used in the RPA was prepared at indicated time points (0, 4 and 8 h) after dopamine treatment. The control shows the RNase digestion pattern of probes in a reaction without cell lysate.

significantly increased *DRD2* mRNA stability for WT but not for 957T (Fig. 3B and C). In the absence of dopamine, 957T again showed a faster mRNA decay rate than WT. These results suggest that the increased amount of *DRD2* mRNA induced by dopamine was probably due to the stabilization of *DRD2* mRNA and, interestingly here, dopamine stabilizes *DRD2* mRNA carrying the 957T mutation significantly less than WT.

Characterization of the folding of mRNA containing synonymous SNPs

Previous study of the *Drosophilia* genes *Adh* and *Adhr* suggests that there maybe a relationship between mRNA secondary structure and gene expression (5,6,29). Therefore, we hypothesized that the observed effects of the synonymous mutations on the properties of *DRD2* mRNA may result from the changes in mRNA folding. Using MFOLD (30), we modeled the effect of the synonymous mutations on the *DRD2* mRNA folding structures of the coding region. Partial mRNA folding structures of WT mRNA and mutant 957T, 1101A, 957T/1101A are shown in Fig. 4. Mutation 957T causes an obvious change in the mRNA folding structures spanning the sequences 804-1212. The mRNA carrying 957T/1101A displays a similar folding structure to WT and mutant 1101A. The folding structures containing other mutations (132T, 423A, 765C and 939T) are all similar to that of WT (data available upon request). The larger effect of 957T on mRNA structure is consistent with experimental data showing a specific effect of 957T on *DRD2* mRNA stability and receptor expression. Taken together, these findings suggest that the *DRD2* mRNA secondary structure may account for the observed differences of *DRD2* mRNA stability and other properties resulted from synonymous substitutions.

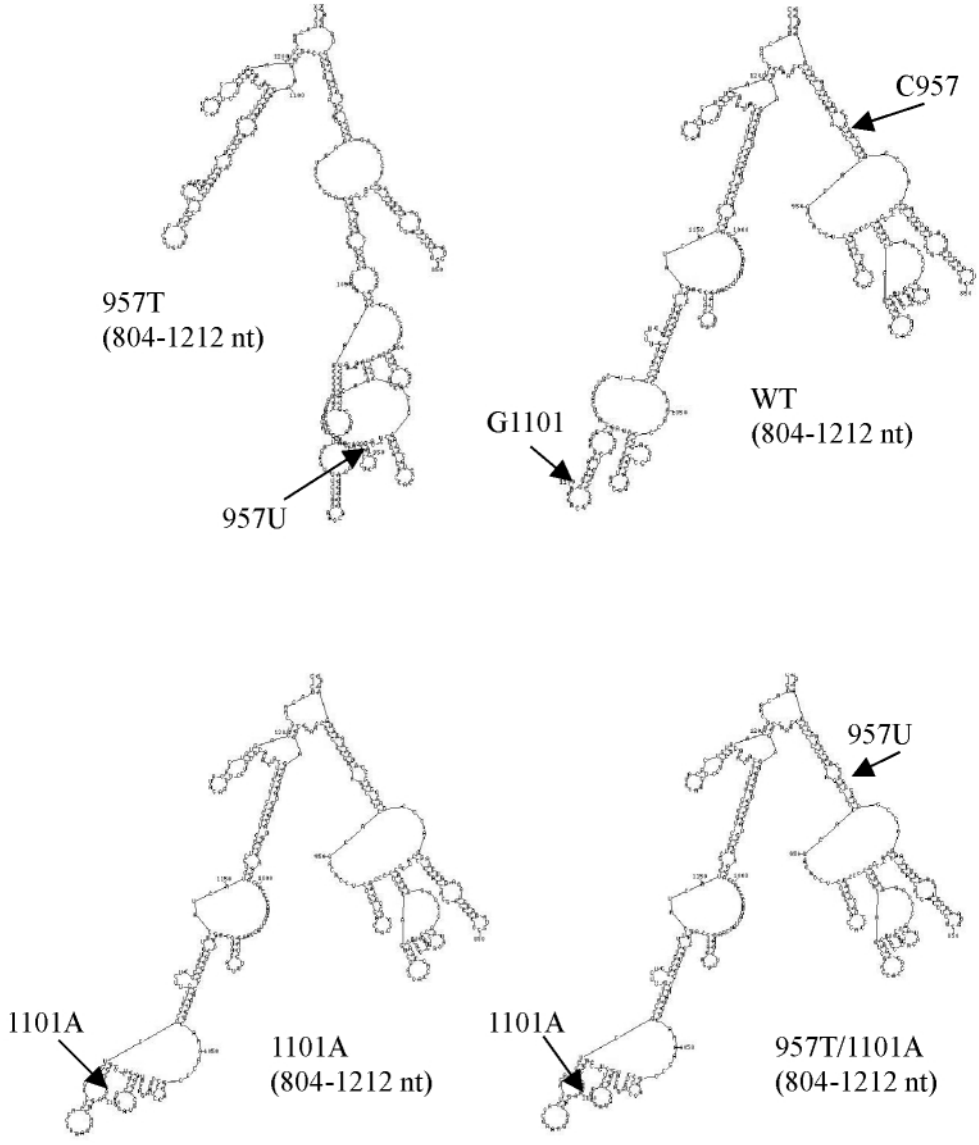

Figure 4. Examples of *DRD2* mRNA folding structures predicted by MFOLD. *DRD2* mRNA sequence carrying various synonymous SNPs was used for secondary folding structure model building by the use of the computer program MFOLD (30). Only part (nt 804–1212 of the coding sequence) of each modeled structure for the *DRD2* mRNA carrying mutations 957T, 1101A, or 957T/1101A, and the WT are shown. Note an obvious change of folding patterns between the WT and the 957T *DRD2* mRNAs.

C957T is in LD with the -141C Ins/Del and *Taq*I 'A' variants

To explore the possible population genetic implications of the functional synonymous mutations 957T and 1101A, we genotyped the C957T and G1101A variants in samples of 94 European-American (EA) and 51 African-American (AA) subjects who had been screened to exclude major psychiatric illness, as previously described (31). We observed allele frequencies of 0.43 and 0.91 for C957 in these two populations (Table 2), respectively. We did not observe any instances of 1101A. In a subset of the subjects (93 EA and 32 AA), we evaluated linkage disequilibrium (LD) with two other *DRD2* markers, -141C Ins/Del and the *Taq*I 'A' system (dbSNP accession number rs1800497), which have been used as markers in previous association studies of *DRD2* and a variety of psychiatric disorders (23). Significant LD was detected in the EA sample between the C957T marker and both the -141C Ins/Del marker ($d = 0.033$, $d' = 0.724$, $G = 7.58$, $P = 0.0088$) and the *Taq*I 'A' marker ($d = 0.071$, $d' = 0.832$, $G = 16.20$, $P = 0.0003$). Significant LD was not detected in the smaller AA sample.

Table 2. C957T genotype counts and allele frequency in EA and AA samples

Sample (number of subjects)	Genotype counts			Allele frequency	
	C/C	C/T	T/T	C	T
EA (94)	18	45	31	0.43	0.57
AA (51)	42	9	0	0.91	0.09

DISCUSSION

For mammalian genes, a translational effect of synonymous sites remains elusive. Some experiments show that altering codon usage to G- and C-ending codons can enhance the expression levels of genes in human cell lines (32,33). There is also evidence for a correlation between tRNA abundance in human and rabbit reticulocytes and the codon usage of α- and β-*globin* mRNAs (34). The selection for translational accuracy of synonymous codon usage in *Drosophila* genes is supported by the correlation between synonymous divergence and amino acid conservation within mammalian genes (35,36). Further, a test for translational selection at silent sites in the human genome revealed that GC-ending codons are more abundant in constitutive than alternatively spliced exons (4). However, other reports showed no relationship between expression patterns and synonymous codon usage in human genes, and no relationship between codon usage bias and synonymous divergence among mammals (37,38).

In this study, we present experimental evidence that the naturally occurring synonymous SNP C957T in *DRD2* can markedly affect the *in vitro* expression of the gene, while the other five synonymous SNPs (C132T, G423A, T765C, C939T and G1101A) were shown to be silent. We have shown compelling evidence that the synonymous mutation 957T in *DRD2* significantly affects mRNA stability. The striking effect of 957T on both *DRD2* mRNA stability and receptor expression is consistent with the changes of predicted mRNA secondary structure (Fig. 4), suggesting that mRNA secondary structure associated with the synonymous mutation 957T may play an important role in the regulation of *DRD2* gene expression. Relevantly, it was shown that two distant synonymous mutations in *ITGB3* [*integrin, beta 3* (*platelet glycoprotein IIIa, antigen CD61*)] were required to perturb pre-mRNA folding and induce altered mRNA splicing (10). It has been proposed that joint effects of mRNA secondary structure and codon bias may interact to regulate the level of gene expression (5). Selection may act on synonymous codons to maintain the structural features of the mRNA that make it more resistant to degradation (5,6,39). The high allele frequency of 957T in the population suggests that the functional C957T dimorphism may not be very deleterious, otherwise strong selection might preclude its observation in the population. This is consistent with a genetic model of complex disease in which mildly deleterious mutations may have quite high allele frequencies (40).

The mechanism underling the observed lower translational efficiency and the decreased mRNA stability for 957T is not obvious. The bulk of mRNA turnover is mediated either by mRNA decapping 5′-to-3′ decay or exosome-mediated 3′-to-5′ exonucleolytic decay (41). While it is possible that 957T impairs translation initiation, typically this is produced by changes in the 5′-UTR sequence, not the coding sequence. According to a general model about mRNA translation efficiency and mRNA decay (42), it is also conceivable (but difficult to test) that 957T causes a global perturbation of mRNP structure, precluding the normal bridging interactions that basically involve 5′-cap structure, eukaryotic translation initiation factor 4E (eIF4E) and eIF4G, poly(A)-binding protein (PABP), and the poly(A)-tail (42). Perturbation of this closed loop conformation predisposes to decapping and the accelerated mRNA decay and also impairs translation efficiency by decreasing recruitment of the 40S ribosome subunit (42,43). It is also possible, although unlikely, that 957T might interfere with the base-pairing structure between 5′- and 3′-UTRs; impaired long-range pairing between the UTRs could conceivably impair the translational efficiency and promote transcript degradation by allowing accelerated decapping (44,45).

The regulation of mRNA decay is an important control point of gene expression, and is mediated by a subset of sequence elements, factors, and endoribonucleases (42). Most of the mRNA stability elements are considered to be located in the 5′- and 3′-UTRs (untranslated regions) of genes. However, we have shown here that a synonymous mutation in the coding region of *DRD2* could affect mRNA stability. Indeed, there is a rapidly expanding literature describing determinants of mRNA stability in the coding region of multiple genes (5,46–50). The mechanisms by which these coding region mRNA stability/instability elements affect mRNA decay are complex. The change of mRNA stability induced by the synonymous 957T in *DRD2* suggests the existence of an mRNA stability/instability determinant flanking or encompassing this locus that affects mRNA conformation. This is supported by the fact that the combined mutations, 957T/1101A, did not result in any changes of mRNA stability. mRNA folding structures predicted by MFOLD (Fig. 4) also support a change of mRNA secondary structure. However, because of the limitation of structure prediction of MFOLD, other RNA secondary structure prediction methods (6) and more accurate assays (*in vitro* or *in vivo*) may be needed to perform additional analyses. Future experimental efforts on the characterization of another synonymous change (not limited to the naturally occurring ones we have investigated) in the vicinity of 957T that causes a similar alteration in the local mRNA folding would provide further evidence to support our interpretation of the data. Furthermore, to test our hypothesis more comprehensively, we are studying whether naturally occurring synonymous mutations affect the performance of transcripts of genes that do not show excess GC3 [e.g. 5-*hydroxytryptamine* (*serotonin*) *receptor 1B* = *HTR1B*] and also plan to test the effect on transcripts harboring a variety of induced (but not naturally occurring) synonymous mutations in *DRD2* with functional assays.

The *DRD2* promoter variant (-141C Ins/Del) has previously been reported to affect *DRD2* mRNA expression and to be associated with schizophrenia (27). The *DRD2 Taq*I ‘A’ marker, a SNP located about 10 kb downstream of the termination codon (51), has also been reported by some investigators to be associated with a range of psychiatric phenotypes, including drug dependence (52) and alcoholism (53), although this has not been replicated (54). The strong LD observed between -141C Ins/Del and the functional synonymous SNP C957T could confound studies unless the functional effects of 957T are considered, and it is possible these two mutations could have additive, multiplicative, or other interactions, though we have not tested this. Furthermore, no common functional variant was previously known that could account for the claimed associations observed with *Taq*I ‘A’. The strong LD observed between C957T and *Taq*I ‘A’ could, finally, provide a molecular basis for the traits attributed to *Taq*I ‘A’.

Interestingly, the most closely related non-human primate full coding *DRD2* sequence (from one chimpanzee, *Pan troglodytes*; GenBank accession numbers AB080603-9 for exons 1–7, respectively) has the same nucleotides as the human WT (i.e. human major alleles) at all of the human polymorphic cSNP sites, including C957T, thus indicating that the C is likely the most recent ancestral form. Several more distantly related species have a T instead of a C at the same position in *DRD2*, e.g. *Cercopithecus aethiops* (African green monkey; GenBank accession number U18547) and *Mus musculus* (house mouse; GenBank accession number X55674), indicating that the more distant ancestral form might be a T, although the very frequent occurrence of C to T mutations during evolution could account for this as well.

1101A did not have any effect by itself, but consistently annulled the effects of 957T in the compound clone 957T/1101A. This suggests another way in which reliance on isolated SNPs, when mapping genes responsible for diseases with complex inheritance, may be insufficient from a functional perspective. It may very well be that combinations of mutations, rather than isolated mutations, are associated with disease. Our demonstration of the interaction between two *DRD2* SNPs bolsters the case for studying SNP combinations at particular candidate genes as opposed to single SNPs, and is consistent with work indicating that interactions of multiple SNPs within a haplotype affect biological traits of the human *beta-2 adrenergic surface receptor* (*ADRB2*) (55). This result is also consistent with a proposed compensatory evolution model involving mutations from two or more loci 56. In that model, single mutations are assumed to be deleterious, but neutral in appropriate combinations. In the case of *DRD2*, 1101A does not have a deleterious effect by itself, and compensates for the effect of 957T in the compound clone 957T/1101A by changing a potential mRNA secondary structure. Relevantly, previous site-directed mutagenesis experiments found that functional effects of synonymous changes in the *Adh* gene of *Drosophila melanogaster* could be compensated by changes in the 3′ -UTR of *Adh* mRNA (6,7). The authors interpreted this observation as evidence for long-range interactions across the *Adh* mRNA secondary structure.

In summary, we report for the first time that the synonymous *DRD2* mutation 957T has empirically measurable biological consequences compared to the WT C957. These include decreased mRNA translation, decreased mRNA stability, and a weakened response to dopamine-induced up-regulation of *DRD2*. We suggest that 957T alters an mRNA secondary structure that underlies these changes, possibly by altering a binding site for a protein factor, a hypothesis that remains to be tested.

MATERIALS AND METHODS

Mutagenesis and plasmid construction

A 2.5 kb cDNA fragment with 5′ and 3′ UTRs encoding the long isoform of the human *DRD2* (17) was subcloned into plasmid cytomegalovirus (pCMV) Script (Stratagene). The resultant pCMV-Script-*DRD2* was used as a template for mutagenesis by overlapping polymerase chain reaction (PCR) with mutagenic primers (sequences are available upon request) to introduce the mutations (subsequently confirmed by DNA sequencing).

GC3 and GC-noncoding analyses

Thirty-five GPCRs were selected, including five dopamine receptors (*DRD 1-5*), 12 serotonin receptors (*HTR 1A, 1B, 1D, 1E, 1F, 2A, 2B, 2C, 4, 5A, 6* and *7*), nine adrenergic receptors (*ADR A1A, A1B, A1D, A2A, A2B, A2C, B1, B2*, and *B3*), five cholinergic receptors (*CHRM 1-5*), and four histamine receptors (*HRH 1-4*). GC3 was calculated from consensus human cDNA sequences while the GC-noncoding (isochoric G + C content) was estimated from flanking (5′ and 3′) noncoding sequences with an average size of 3.5 kb (Fig. 1). The comparison between coding and noncoding sequences of *DRD2* and those of genes immediately flanking it was conducted using the chromosome 11 reference genomic contig NT_035088.1 (8.3 Mb), and only flanking genes with mRNA evidence were used in the analyses. The following coding sequences were used as immediately flanking *DRD2*: centromeric–*LOC143914–FLJ20535–LOC143915–3′_DRD2_5′–LOC143916–TMPRSS5–ZW10*–telomeric. In this intra-isochoric analysis, three different sizes of noncoding 5′ sequence were used: 1 kb 5′ to the start codon, and 1 kb and 3 kb 5′ sequence after removing potential 5′UTR regions assuming an average size of 300 bp (28).

Cell culture, cell transfection, and drug treatment

CHO-K1 cells (American Type Culture Collection) were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C. CHO-K1 cells do not endogenously express *DRD2*, and are a commonly used model to study *DRD2* gene expression and regulation (26,57,58). Stable transfections of plasmids into CHO-K1 cells were performed using the LipoTAXI mammalian transfection kit (Stratagene). Transfected clones were selected in medium supplemented with 800 μg/ml of G418, and resistant colonies (>300) were pooled by trypsinization for further study. Expression of the receptor on transfected cells was studied by radioligand binding. The sequence of *DRD2* in stable transfectants was confirmed by RT-PCR followed by sequencing. For the mRNA stability assays, ActD (10 μg/ml) was applied to the cell cultures grown overnight and cells were harvested at selected intervals up to 24 h. For the dopamine regulation experiments, the cell cultures grown overnight were treated with dopamine (100 μM) in the presence of 0.2 mM sodium metabisulfite (to prevent oxidization of dopamine in the culture medium), and cells were harvested at selected intervals up to 24 h.

In vitro translation

mRNAs used for *in vitro* translation were generated by *in vitro* transcription using plasmids linearized downstream of the respective *DRD2* inserts with *Hin*dIII as template. The MEGAscript T3 kit (Ambion) was used according to the manufacturer's instructions. To control the quality and quantity of input mRNA, the *in vitro* transcribed mRNAs were checked

for integrity by electrophoresis on formaldehyde agrose gels followed by quantification of concentration and purity by spectrophotometry using the GeneQuant *pro* RNA/DNA Calculator (Amersham). The concentrations of transcripts were typically 2.424 ± 0.151 μg/μl. Subsequently, mRNA samples were diluted to equal concentrations (1 μg/μl) and 1.5 μl of diluted mRNA was added to the 25 μl translation reaction. *In vitro* transcribed mRNA were translated in the Flexi® rabbit reticulocyte lysate system (Promega) in the presence of biotin-labeled Transcend™ tRNA (Promega) between 0 and 60 min at 30°C. Following electrophoresis and electro-blotting, the Transcend™ Non-Radioactive Translation Detection System (Promega) was used to detect protein synthesis.

RPA (RNase protection assay)

Detection and quantitation of *DRD2* mRNA in transfected cells was carried out using the Direct Protect Lysate RPA kit (Ambion) with *ACTB* as an internal control. Forty microliters of cell lysate in Lysis/Denaturing solution from 5×10^5 cells were directly used in each RPA reaction, and 5 μl ^{32}P-labeled *DRD2* RNA probe and 5 μl ^{32}P-labeled *ACTB* RNA probe were added to each reaction. The band intensities were quantitated by Typhoon 8600 (Amersham Pharmacia). The RNA probes used in RPA were synthesized using MAXIscript T7 (Ambion) and labeled by [α-^{32}P]UTP (Amersham Pharmacia). For the *DRD2* RNA probe, a PCR-generated 310 bp DNA fragment of *DRD2* with a T7 promoter added by the No-cloning Promoter Addition Kit (Ambion) was used as the template to generate the RNA probe. For the *ACTB* RNA probe, a plasmid TRIPLEscript™–*ACTB* (Ambion) was used as template to make an antisense mouse *ACTB* RNA 304 bp probe. The sizes of the expected protected fragments were about 310 and 250 bp for *DRD2* and *ACTB*, respectively. ^{32}P-labeled RNA Century Marker (Ambion) was used as a size marker.

Radioligand binding assays

[^{3}H]methylspiperone (New England Nuclear) was used to label receptor binding sites (26,57). Disruption of the cells was performed using a Sonic Dismembranator. Cell membranes were collected by centrifugation at 34 000 *g* for 20 min at 4°C and resuspended in binding buffer at a final protein concentration of 0.3 mg/ml. Membrane suspension (100 μl) was added to triplicate assay tubes containing [^{3}H]methylspiperone (0.01–1.2 nM) in a final volume of 1 ml. Non-specific binding was defined using 1 μM (+)-butaclamol. The assay tubes were incubated for 1 h at 25°C. The assay was terminated by rapid filtration through GF/C filters (Whatman) pretreated with 0.3% polyethylenimine. The filters were rapidly washed three times with 4 ml of 50 mM Tris–HCl, pH 7.4, at 4°C, and the radioactivity bound to the filters was quantitated by scintillation counting. Protein concentrations were determined using the bicinchoninic acid reagent (Pierce). Radioligand binding data was analyzed with the non-linear least-squares fitting program GraphPad Prism version 3.0 (GraphPad Software, Inc.).

Genotyping, LD analysis, and sequencing

Genotyping was performed as previously described (31). We designed a PCR restriction fragment length polymorphism assay to detect the C957T and G1101A variants. Detailed genotyping design is available upon request. The amplicon includes both C957T and G1101A, so in dually heterozygous subjects molecular haplotyping is possible via double digestion. LD was analyzed by the program 3LOCUS (59) and *P*-values were estimated with 10 000 Monte Carlo simulations. Sequencing of the chimpanzee *DRD2* was performed using standard methods in the laboratory of N.S.

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