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Post-Genomic Era and Gene Discovery for Psychiatric Diseases: There Is a New Art of the Trade?

The Example of the HUMTH01 Microsatellite in the Tyrosine Hydroxylase Gene

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Abstract

The microsatellite HUMTH01, located in the first intron of the Tyrosine Hydroxylase (TH) gene (encoding the rate-limiting enzyme in the synthesis of catecholamines), is characterized by a TCAT repeated motif and has been used in genetic studies of neuropsychiatric and other complex diseases, in which catecholaminergic neurotransmission is implicated. After reporting a positive association between HUMTH01 and bipolar disorder as well as schizophrenia, the authors established that HUMTH01 alleles display the features of regulatory elements. Thereafter, they cloned two proteins (ZNF191 and HBP1), specifically binding to HUMTH01, and demonstrated that allelic variations of HUMTH01 have a quantitative silencing effect on TH gene expression in vitro, and correlate with quantitative and qualitative changes in the binding by ZNF191. The authors aim to characterize the transduction pathway impinging on the HUMTH01 microsatellite and establish its relevance for TH gene regulation in vivo. Since the TCAT repeated sequence is widespread throughout the genome, their approach may lead to the dissection of the mechanisms underlying the quantitative expression of several genes implicated in complex genetic traits, both normal and pathological. Thus, these investigations on the possible contribution and potential role of the HUMTH01 microsatellite in neuro-pathological conditions may represent an example of the different approaches needed to validate genetic targets in the "post-genomic era."

Index Entries: Tyrosine hydroxylase; bipolar disorder; schizophrenia; genetic studies; functional studies; microsatellites; quantitative genetic traits.

Introduction

The progress made over the past 20 yr in the field of genetics is based on the positional cloning technique that has completely revolu-

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Table 1
Genetic Characteristics of Rare and Common Diseases

Rare disease	Common disease
Single mutation	Several polymorphisms
High penetrance:	Low penetrance:
No modifying genes	Modifying genes
No environmental factors	Environmental factors.
Present only in affected or carrier subjects	Present also in non-affected subjects in the general population
Recent genetic event	Ancient genetic event
Mode of transmission established	Mode of transmission uncertain
Detection straightforward by parametric linkage studies	Not amenable to detection by parametric linkage studies
Qualitative trait	Quantitative trait

tionized the task of identifying genes involved in human hereditary diseases. Positional cloning allows for the localizing of the genetic defect responsible for a disease without prior knowledge of the protein or the physiological pathway involved. Its application is based on the utilization of polymorphic genetic markers.

Positional cloning was initially based on meiotic or linkage mapping using the lod score method to discover the causative mutation for rare monogenic diseases in previously unknown genes. The lod score is a powerful tool that allows analysis of the co-segregation of a disease by employing genetic markers (linkage) to calculate the likelihood of linkage compared to that of non-linkage in large multigeneration affected families. The application of this method has led to the discovery of the etiology of diseases caused by mutations in a single gene, such as muscular dystrophy, cystic fibrosis, Huntington's disease, etc. To date, more than 120 genes mutated in monogenic diseases have been cloned.

This successful application of the lod score method for positional cloning has raised the hope that it will also allow for the identification of those genes causing predisposition to genetically complex diseases. These diseases are common, highly widespread, have a high socio-economical impact, and include such affections as neuropsychiatric, cardiovascular, and metabolic diseases as well as certain types of cancer. These pathologies "run in families," and several twin, adoption, and family segregation studies have clearly established that they are partly genetic. However, contrary to rare monogenic diseases, these more common diseases are caused by the interplay between multiple genetic and environmental factors. Moreover, heterogeneity and probably epigenetic phenomena also characterize the genetic component. Thus, the genetic factors predisposing to such common diseases are polymorphic variations of the genetic code with modulating effects on gene expression, rather than classical rare mutations with a clear-cut effect (*see* Table 1).

Therefore, more powerful approaches for the positional cloning of common disease genes (different, complementary, and/or alternative) are needed since the genetic components of these complex traits are elusive. Such approaches are based on the utilization of different types of patient recruitment in selected populations to be analyzed with new nonparametric linkage and association (linkage disequilibrium) methods. Prior to the genetic map of man being completed, association studies based on linkage disequilibrium in case/control studies were limited to single loci and/or genes. However, in the near future, with the increasing number of marker and

genotyping efficiency and speed, it will be possible to identify target genes by means of linkage-disequilibrium screening of the whole genome in case/control cohorts.

The recent completion of the genomic map will further facilitate the task of locating genes implicated in complex diseases, while the advent, in the so-called "post-genomic era," of transcriptomic and proteomic approaches will allow for the elucidation of whole disease-related pathways and identification of an increasing number of therapeutic targets. This, in turn, will result in an increased interest in pharmacogenomics and the implementation of a personalized medicine that will shape the future of health care.

However, the results obtained to date for complex diseases, in particular those for the genetics of psychiatric diseases, demonstrate that the transition from a candidate gene to the pathophysiological mechanism underlying the disease will not be straightforward. This research is still hampered by the uncertainties that characterize the correspondence between genotype and phenotype, and the necessity of a functional validation of the disease-related genetic variations for a genomics-based approach to the diagnosis and treatment of psychiatric, as well as other complex diseases.

In this context, the extensive study of the tyrosine hydroxylase (TH) gene offer a paradigmatic example of the difficulties and pitfalls of the research in psychiatric genetics. However, at the same time, TH gene investigations have shed light on complementary approaches that may allow us to overcome these difficulties and validate already known, or newly discovered, genes as putative targets for therapeutic interventions.

The TH Gene and Psychiatric Genetic Studies

The TH gene codes the rate-limiting enzyme in the synthesis of catecholamines (dopamine, adrenaline, and noradrenaline). Neuroanatomical, electrophysiological, and pharmacological data have shown that dopamine (DA) is implicated in the etiology of Parkinson's disease *(1)* and strongly suggest its implication also in psychiatric diseases such as bipolar disorder *(2)* and schizophrenia *(3)*, as well as substance abuse *(4)*. In addition, a mutation in the TH gene was recently linked to Segawa's syndrome, a progressive form of Dopa-responsive dystonia *(5)*. Thus, the TH gene is a strong candidate for neuropsychiatric diseases *(6)*.

Moreover, a seminal paper showing for the first time a genetic linkage between bipolar disorder in the Amish population, and markers at the chromosome 11p15, a region that contains the TH locus, strengthened the case for TH as a "positional" candidate gene *(7)*. This result was questioned because the lod score method used did not take into account genetic heterogeneity which characterizes bipolar disorder and other complex diseases *(8)*. In addition, further genetic analysis of the Amish, or studies of other populations, have not confirmed this initial result *(9–15)*. On the other hand, other studies also found significant linkage between markers at the TH locus and bipolar disorder, thus leaving open the question about the implication of the TH gene in this disease *(16–21)* (*see* Table 2).

The author's laboratory is the first to show a significant genetic association between restriction-fragment polymorphism markers flanking the TH gene, and bipolar disorder in the French population using a case-control study—a non-parametric method more compliant with genetic investigations for complex diseases than the lod-score method *(22)*. However, even with this non-parametric approach, this result has not always been replicated in other studies *(23–27)*.

The TH Gene and the HUMTH01 Microsatellite: Genetic Association Studies

In order to further investigate the implication of the TH gene in the genetic predisposition for

Table 2
Positive Linkage Studies Between the TH Locus and Bipolar Disorder

Author	Journal	Year	Ref.
Egeland et al.	*Nature*	1987	*(7)*
Pakstis et al.	*Hum. Genet.*	1991	*(16)*
Byerley et al.	*Hum. Hered.*	1992	*(17)*
Lim et al.	*Am. J. Med. Genet.*	1993	*(18)*
Gurling et al.	*Nature Genetics*	1995	*(19)*
Smyth et al.	*Am. J. Psychiatry*	1996	*(20)*
Malafosse et al.	*Neurobiol Dis.*	1997	*(21)*

bipolar disorder, the authors used a new sample of case-controls, and a new and more informative marker: the microsatellite HUMTH01—a polymorphic polypyrimidine sequence characterized by a (TCAT)n tetranucleotide repeat localized in the first intron of the TH gene. The iteration of the core (TCAT)n motif has *n* values usually spanning between 6 and 10 *(28, 29).* France was arbitrarily divided into 5 regions, 64 patients, and 64 controls, all of French origin for more than two generations and with at least 3 grandparents of the same geographical origin were matched pair-wise. The (TCAT)7/(TCAT)10 genotype was significantly more frequent among bipolar patients than controls. Moreover, the patients bearing this genotype were characterized clinically by having familial history of bipolar disorder and/or delusive symptoms during manic or depressive episodes *(30).*

Another significant genetic association between the TH gene and schizophrenia using the HUMTH01 microsatellite was obtained. Although the TH gene is an even stronger candidate gene for schizophrenia than for bipolar disorder, there was no compelling evidence for linkage of the TH locus to schizophrenia *(31).* Interestingly, the HUMTH01 microsatellite exhibits a deletion of a single base in the fifth core repeat of its ten tetranucleotide repeat allele. This deletion constitutes an imperfect repeat allele $(TCAT)_{101}$ which is the most common allele in the Caucasian population, whereas the corresponding perfect repeat $(TCAT)_{101}$ that is its non-deleted form, is very rare in most populations studied *(32).* In the bipolar disorder association study the authors found only the imperfect variant of the ten-repeat allele. However, in two different ethnic samples of unrelated chronic schizophrenic patients and unaffected controls from Normandy (Northwestern France) and the Sousse region (Eastern Tunisia), the perfect repeat, albeit rare, was found in the schizophrenic patients, both in the French and Tunisian population. This result yielded for the first time, to our knowledge, a significant association between TH and schizophrenia *(33).*

Moreover, a subsequent clinical study suggests that the perfect HUMTH01 10 repeat polymorphism may be associated with disturbances of the catecholaminergic pathway. Clinical parameters evaluated with the "Positive And Negative Symptoms Scale" (PANSS) and plasma measurements of homovanillic acid (HVA, an index of central dopaminergic function) and 3-methoxy-4-hydroxy-phenylglycol (MHPG, an index of central noradrenergic function) were analyzed in a sub-group of the Normandy sample of schizophrenic patients. While no clinical differences were observed among patients bearing this rare allele compared to those who did not possess this allele, the mean concentration of plasma HVA and plasma MHPG were significantly lower in the group of schizophrenic patients sharing the rare allele *(34).*

Table 3
HUMTH01 Positive Association Studies

Phenotype	Author	Journal	Year	Ref.
Bipolar Disorder	De Castro et al.	*J. Med. Genet.*	1995	*(41)*
	Serretti et al.	*Am. J. Med. Genet.*	1998	*(43)*
	Serretti et al.	*Mol. Psychiatry*	1998	*(44)*
Schizophrenia	Wei et al.	*Psychiat. Genet.*	1995	*(47)*
	Wei et al.	*Life Sci.*	1997	*(48)*
	Kurumaji et al.	*J. Neural. Transm.*	2001	*(49)*
Hypertension	Sharma et al.	*Hypertension*	1998	*(52)*
	Jindra et al.	*Blood Press.*	2000	*(53)*
Diabetes	Hearne et al.	*Trends Genet.*	1992	*(50)*
Diabetes and Depression	Chiba et al.	*Metabolism*	2000	*(46)*
Suicide	Persson et al.	*Psychiatry Res.*	2000	*(54)*
Alcoholism	Sander et al.	*Psychiat Genet.*	1998	*(56)*
Personality traits	Persson et al.	*Psychiatry Res.*	2000	*(57)*
Longevity	De Benedictis et al.	*Eur. J. Hum. Genet.*	1998	*(55)*

While the results of some groups have not always been straightforward *(35–40)*, several other studies have replicated the positive association between the HUMTH01 microsatellite and both bipolar disorder *(21,41–46)* and schizophrenia *(47–49)*. Moreover, the microsatellite HUMTH01 has also been associated with diabetes *(50,51)* because of the proximity of the TH locus to the insulin gene, with arterial hypertension, which is characterized by the implication of catecholamines *(52,53)*, and other complex traits *(46,54–57)* (*see* Table 3).

The Biological Role of Repeated Sequences

With the perspective of cutting through the Gordian knot represented by the contrasting results obtained from population-based genetic association studies with the HUMTH01 microsatellite, the authors have evaluated the biological substrate of this association. Thus, the authors tested the hypothesis that the HUMTH01 microsatellite is implicated in the regulation of the TH gene expression.

Microsatellites *(58)* are highly polymorphic randomly repeated sequences, usually constituted by di- tri- and tetra-nucleotide motifs which are distributed throughout the genome in eukaryotes *(59,60)*. Their considerable polymorphism is due, not only to differences in the number of repeat units but also in some instances to variations in the sequence of the core motif *(61)*—characteristics that make them useful markers for genetic analysis *(50,60)*. Microsatellites, according to the "neutral hypothesis" of their origin, do not have a generalized function *(60)*, although it appears that in many cases they are under evolutionary control *(62)*. However, as shown by the pathological expansion of repeated triplets, microsatellite can interfere with gene expression and be responsible for several hereditary neuro-degenerative diseases *(63)*. In contrast, normal allelic microsatellite variations may be implicated in gene transcription without deleterious consequences for the phenotype. Based on these observations, an hypothesis has been advanced suggesting that microsatellites act as a source for the creation and maintenance of the variability of quantitative genetic traits *(64)*. An extreme manifestation of these traits

may result in frequent and genetically complex diseases *(65).*

In this framework, dinucleotide and trinucleotide repeats have been shown to regulate transcription in vitro *(66,67).* Minisatellites, the other main class of repeated sequences used as genetic markers *(68),* may also be endowed with regulatory functions. For example, the minisatellite located 1 Kb downstream from the HRAS1 proto-oncogeneis associated with increased risk of cancer *(69),* and the minisatellite 5′ from the insulin gene is associated with insulin-dependent diabetes mellitus (IDDM)*(70);* both display transcriptional regulatory activity in vitro *(71–73).* Thus, the capacity to behave as transcription regulatory elements appears to be a general feature of all classes of repeated sequences *(64).*

The HUMTH01 Microsatellite: Functional Studies

Some features of the HUMTH01 microsatellite support the hypothesis that the HUMTH01 microsatellite is implicated in the regulation of the TH gene expression. For instance, the HUMTH01 microsatellite core motif (TCAT)n is very similar to the TPA Responsive Element (TRE) canonical consensus sequence (TGACTCA) *(74,75),* and differs by only one nucleotide from the consensus TRE sequence (TGATTCA) present in the rat and human TH gene *(76).* The TRE site is a sequence that is specifically recognized by the AP1-type transcription factors which results from the auto- or hetero-dimerization by members of the Fos and Jun proto-onco-gene families. Moreover, a less polymorphic HUMTH01 repeated sequence, and its flanking sequences, are conserved at their orthologous position in the first intron of the TH gene in several non-human primate species *(77),* hinting that this motif may be an evolutionary conserved regulatory element.

Using transient transfection studies in three different cell lines, the authors initially investigated the perfect and imperfect variant of the ten repetition allele of the HUMTH01 microsatellite that were the alleles previously associated to schizophrenia and bipolar disorder, respectively. These elements were placed upstream of the Herpes virus thymidine kinase minimal promoter driving the expression of the luciferase reporter gene, and led to up to a tenfold increase of basal transcription in all cell lines. Moreover, as expected for a regulatory element, this effect was not dependent on the orientation of the sequences *(78).*

In a second set of experiments, it was shown that nuclear proteins bind in a sequence specific manner to stretches of this tetrarepeat. Two major complexes were detected. Since the motif (TCAT)n is very similar to the TRE canonical consensus sequence, the authors tested competition between the ten repetition perfect and imperfect alleles, and the TRE consensus sequences. One of the complexes was abolished in a dose-dependent fashion by competition with a canonical TRE consensus sequence as well as the TRE consensus sequence present in the rat and human TH, suggesting that members of the Fos-Jun family form this complex. However, the second complex formed was not affected by the competition by TRE, suggesting that factors other than those of the Fos-Jun family are able to bind with greater affinity to the HUMTH01 microsatellite motif. The proteins responsible for the formation of this second complex, may represent a novel class of unidentified regulatory factors *(78).*

These results, suggesting for the first time that this tetranucleotide repeat is a novel regulatory sequence whose action may be relevant to gene expression, prompted the authors to determine whether the HUMTH01 microsatellite acts as a transcription enhancer of the TH gene. Thus, the role of the HUMTH01 microsatellite at its orthologous position in the context of the TH gene was examined further. Several different types of constructs containing the sequence of the human TH gene from –2187 to +1300, including the distal promoter, 3 exons and 2 introns, fused to the luciferase as a reporter gene were used. To test how the transcriptional activity related to the number of

repeats, constructs $(TCAT)_0$ $(TCAT)_3$ $(TCAT)_5$ $(TCAT)_8$ $(TCAT)_{10p}$ and $(TCAT)_{10i}$ were generated and investigated by transient transfection into catecholaminergic cell lines.

Quite interestingly, it was found that the HUMTH01 microsatellite regulates TH gene transcription and acts as a quantitative genomic effector modulating the activity of the TH gene. In their orthologous position in the first intron of the TH gene, the HUMTH01 alleles inhibited transcription in a stepwise mode proportional to the number of repeats from 3–8. However, increasing the number of repeats from 8–10 perfect or imperfect repetitions did not produce any further inhibitory effect; rather it resulted in no changes, or in a relative enhancement of transcription depending on the cell line used. A different repeated sequence with a tetranucleotide repeat motif did not affect the reporter gene expression, demonstrating that the effect on transcription was specifically due to the $(TCAT)_n$ sequence. Interestingly, the longer sequence encountered in non-human primates corresponds to 8 repeats. The presence of longer alleles, and the fact that the most frequent and longer allele in humans presents a point mutation that prevents further expansion, would strengthen the fact that the HUMTH01 microsatellite is endowed with a functional role *(79).*

The HUMTH01 alleles act as enhancers when placed upstream of the minimal promoter of the thymidine kinase gene, whereas they are transcriptional repressors when placed in the first intron of the TH gene. Thus, the effects of the HUMTH01 microsatellite depend on the presence of other sequences in the TH gene. However, the substitution of the 5′ regulatory region of the TH construct with a collagenase gene minimal promoter did not affect the profile of transcriptional regulation exerted by the $(TCAT)_n$ sequence. Furthermore, the activity of the $(TCAT)_n$ sequence is independent of both its orientation and its position relative to the flanking sequences, strongly suggesting that the repeated sequence acts independent of 5′ promoter regulatory elements, and is not dependent on orientation or physical continuity with adjacent flanking sequences. However, these results do not exclude the involvement of elements in the first intron or its flanking exons in the HUMTH01 microsatellite activity *(79).* In this context, the evaluation of the methylation profile of the CpG island that encompasses the promoter and the first exon and intron of the TH gene has allowed the characterization of a sequence differently methylated between TH positive and TH negative cell lines. Preliminary studies indicate that this sequence may represent a novel consensus site for TH gene regulation that may act in concert with the HUMTH01 microsatellite (unpublished data).

Identification and Characterization of Proteins Interacting with the HUMTH01 Microsatellite

To isolate the protein(s) interacting with the (TCAT) repeat, the authors set up a yeast one-hybrid system (ref. *16*) using the $(TCAT)_{10i}$ as bait for screening a human brain cDNA library. One hundred clones were isolated based on their ability to induce HIS3 transcription placed under the control of 2 $(TCAT)_{10i}$ motifs. The most abundant cDNA (70%), which also had the largest effect in the one-hybrid system, corresponds to the zinc finger protein ZNF191 that was previously cloned from hematopoietic cells *(80).* ZNF191 is a putative transcription factor belonging to the Kruppel family and containing four C2H2 zinc fingers in its C terminal, and a SCAN box element upstream of the zinc-finger domain. The SCAN box is a highly conserved interaction domain allowing homologous and heterologous oligomerization of the members of this family of transcription factors *(81).* The second most abundant clone (20%) in the yeast one-hybrid screen corresponds to HBP1. HBP1 is a HMG box transcription factor with a chromatin remodeling role in the LCR (locus control region) of the CD2 gene, where it specifically binds a TTCATTCATTCA sequence, including the (TCAT) motif *(82).*

The authors further investigated ZNF191 since it was encoded by the most abundant cDNA identified by, and had a stronger effect in, the one-hybrid system. The ZNF191 transcript is abundant in heart, lung, and spleen but it is also represented in uro-genital tissues (kidney, testis, prostate) and lymphatic tissues (lymph node, thymus, and tonsil). Low levels of ZNF191 transcript were also detected in the brain, which may reflect dilution of specific expressing regions in total tissue. In order to ascertain the distribution of ZNF191 mRNA in the central nervous system (CNS) we probed the rat brain structures using the human sequence. The ZNF191 transcript was present at high levels in catecholaminergic tissues, including the substantia nigra, the hypothalamus, and the olfactory bulb. It was less abundant in the striatum, the cortex, and the cerebellum. Interestingly, the transcript was also abundant in the adrenal medulla, a peripheral catecholaminergic tissue.

This was followed by a testing of the transcriptional activity of ZNF191 using a functional assay with two kinds of reporter constructs having the (TCAT) repeats upstream of the minimal thymidine kinase promoter and the luciferase gene, or in the first intron of a TH-luciferase fusion gene. These were thus tested in ZNF191 contransfection experiments in PC12 cells. Cotransfection with ZNF191 and the $(TCAT)_n$ repeat in its native intronic context resulted in transcription, indistinguishable from that in the absence of ZNF191. This would suggest that in the context of the TH gene, either the effect of the ZNF191 gene is already maximal, or its interaction with the HUMTH01 sequence is in competition with other factors. However, the cotransfection with ZNF191 significantly increased the enhancing effects of the $(TCAT)_n$ sequence on the expression of a minimal thymidine kinase-luciferase construct—evidence of a functional interaction between ZNF191 and the $(TCAT)_n$ repeat *(79).*

The authors ascertained the specifics of the interaction between ZNF191 and the (TCAT) repeated sequence using the purified protein and the $(TCAT)_n$ sequences, with *n* spanning from 3 to 10_p and 10_i, as probes in electrophoretic mobility shift assay (EMSA). ZNF191 specifically interacted with all the probes yielding either one or two complexes according to the number of $(TCAT)_n$ repeats. UV cross-linking experiments demonstrated that the complexes corresponded to the probe bound to one or two molecules of ZNF191, respectively. The complex with higher mobility, corresponding to the binding of a second ZNF191, was not observed with probes shorter than $(TCAT)_6$.

This fact was further confirmed by footprint experiments used to determine the binding site of ZNF191 within the $(TCAT)_n$ repeated sequences. Shorter probes had one binding site for ZNF191, whereas the probes $(TCAT)_8$ $(TCAT)_{10i}$ and $(TCAT)_{10p}$ had two binding sites. In all cases, the protected region covered ~3–4 repeats showing that the minimal binding site is a $(TCAT)_3$ motif. As ZNF191 has four zinc fingers, it is possible that each finger binds one (TCAT) motif. For $(TCAT)_{10i}$ and $(TCAT)_{10p}$ probes, a central region between the two sites was not protected. The differences in the protection profiles of the $(TCAT)_{10i}$ and $(TCAT)_{10p}$ probes suggest that the presence of an imperfect motif renders these sequences non-equivalent for the ZNF191 binding. Interestingly, the two sites in these longer probes were also not equally protected, suggesting that the affinities of ZNF191 for these two sites were not identical. Effectively, the authors determined by Scatchard analysis, that all probes had one identical binding site with a high affinity at the 3′ site of the $(TCAT)_n$ sequence, while the probes with longer repeats exhibited a second binding site in 5′ with lower affinity *(79).*

New Genetic Association Studies with the HUMTH01 Microsatellite

The discovery of a functional role for quantitative gene expression of the HUMTH01 has had an instant fall-out with the interpretation of some data gathered by the authors, and the design of a more adaptable test for their

interpretation. In fact, in an association study in 100 triads (bipolar proband with both parents) from Sardinia, the authors observed a trend toward increased relative risk in response to increased length of the HUMTH01 alleles. Their objective was to evaluate whether this trend reflected a phenotypic manifestation of the functional effects of the HUMTH01 microsatellite. Since the available family-based association tests, such as the Transmission Disequilibrium Test (TDT), do not allow the evaluation of such a trend, the authors developed a new test, named trend-TDT to assess the significance of a linear trend across allele lengths. The trend-TDT is a paired t-test based on the quantitative value assigned to the length of alleles transmitted from the heterozygous parents to their affected children. The application of the trend-TDT in the Sardinian triads revealed a significant association between the elongation of the HUMTH01 alleles and bipolar disorder *(83).* The application of this method to another sample of more than 100 Egyptian triads genotyped, using the HUMTH01 microsatellite, yielded a similar result after taking into account the differences in the marker allelic frequencies between populations (unpublished results).

Role of Polypyrimidine Sequences of the $(TCAT)_n/(UCAU)_n$ Type

Collectively, the authors'findings on the genetic association of the HUMTH01 microsatellite with psychiatric diseases and its role on gene regulation provides a strong indication that it may act as a quantitative genomic effector determining the expression of traits associated with the activity of the TH gene. It has been proposed, mostly on theoretical grounds, that repeated sequences may be implicated in the expression of quantitative genetic traits *(64).* As the $(TCAT)_n$ repeats and other polypyrimidine tract polymorphic motifs are widespread in the genome, and present in other genes, the associated control mechanisms is likely to be relevant to phenotypic manifestations of a variety of quantitative genetic traits.

Interestingly, the TH gene is closely linked to the insulin gene *(84)*. Studies on the genetics of insulin-dependent diabetes mellitus (IDDM), another complex disease, show the association between a minisatellite (Ins-VNTR) located 5' from the insulin gene and the disease *(70).* The polymorphism causing IDDM has not been definitively identified, although several studies have shown an association between the HUMTH01 microsatellite and diabetes *(50),* or between haplotypes extending to the TH locus and diabetes *(51).* The Ins-VNTR can regulate transcription in vitro, and thus, it has been suggested that it can act on the expression of the insulin gene *(72,73),* as well as on the expression of other closely linked genes, such as the TH gene *(72).* Conversely, the authors' results indicating that the TH tetrarepeat acts as a transcription regulator raises the possibility that the HUMTH01 microsatellite might exert some activity on the expression of nearby genes such as the insulin gene.

Moreover, the T(TCAT)2TCA sequence, present in the LCR of the CD2 gene, is specifically recognized by the HBP1 transcription factor and has been implicated in gene regulation *(82).* Another similar role is played by a polymorphic $(CCAT)_n$ tetranucleotide polypyrimidine stretch that is able to inhibit the "in vitro" transcription of the CD30 gene. This gene codes a receptor for the tumor-necrosis factor and is over-expressed in several lympho-proliferative diseases *(85).* More recently, the polypyrimidine sequence of a polymorphic pentanucleotide microsatellite in the promoter of the PIG3 (p53-induced gene 3) has been shown to be specifically recognized by the p53 protein. The binding of p53 to this sequence induces the transcriptional activation of the PIG3 gene that is correlated to the number of repeats *(86).* Finally, another repeated sequence of the $(CATA)_n$ type is associated with marked behavioral differences in two species of mouse vole. The

presence of this sequence in the vasopressin V_{1a}-receptor gene is the only difference conditioning the social behavior (monogamy, sociality, and paternal care versus promiscuity, asociality and, lack of paternal care) between the prairie and the mountain mouse vole species *(87)*.

Interestingly, the $(UCAU)_n$ sequence corresponding to the $(TCAT)_n$ polypirimidine tract at the mRNA level, plays another regulatory role that has been discovered by studies on the Paraneoplastic Opsoclonus Myoclonus Ataxia (POMA) *(88)*. The POMA is part of paraneoplastic syndromes that are characterized by pulmonary or gynecological cancers, expressing in an ectopic manner several neuronal antigens. These antigens evoke an immunity response producing antibodies directed simultaneously against the cancer cells and some specific types of neurons *(88)*. The Ri antibody causing the abnormal motor control of the trunk, limbs, and eyes is directed against a 55 Kd nuclear neural antigen corresponding to the Nova protein *(89)*. Two forms of Nova have been found: Nova-1 and Nova-2 which are specifically expressed in the brain. Nova-1 expression is limited to the hypothalamus, ventral mesencephalon, hindbrain, and spinal cord—a distribution that may explain several of the POMA symptoms *(89)*. Nova-2 is expressed in the cerebral cortex and thalamus, and is implicated in a different form of POMA characterized by focal neurological deficits such as encephalopathy, dementia, and cerebral atrophy *(90)*. Nova is homologous of hnRNPK (heterogeneous nuclear ribonucleoprotein K), a mRNA-binding factor that is implicated in alternative splicing in the brain *(91)*. Nova and hnRNPK are characterized by a KH (K homology) domain that is present in several other proteins interacting with the mRNA, such as FMR1, that bind to polypyrimidine tracts *(92)*. Nova-1 binds to the polypyrimidine sequence $(UCAU[N]_{0–2})_3$ *(93,94)*, which is present in the GABA-A receptor gene (whose alternative splicing is regulated by Nova-1), the Glycine-alpha2 receptor, and also the Nova-1 gene itself *(88)*.

Future Perspectives

Interestingly, the HUMTH01 microsatellite is localized in the first intron, a region that regulates the alternative splicing of the TH gene *(6)*. Thus, the possible implication of the sequence $(TCAT)_n/(UCAU)_n$ in the control of gene expression, either at the transcription or alternative splicing level, may open new perspectives in human genetic studies. Therefore, the study of the mechanism of action of the HUMTH01 microsatellite in vivo is of paramount importance for psychiatric diseases.

Recently, it has been shown that peripheral lymphocytes are able to release DA *(95)* in way that can be pharmacologically modulated *(96)*. Moreover, they present on their surface the DA receptors DR3, DR4 *(97)*, DR5 *(98)*, and the DA transporter (DAT), as well as the vesicular monoamine transporter (VMAT) *(99)*. Another study, evaluating the expression of the DR3 receptor in lymphocytes from schizophrenic patients, has revealed a profile similar to the variations found in postmortem schizophrenic brains *(100)*. In a preliminary study, the authors have established the presence of the TH mRNA in human lymphocytes (unpublished data). The authors are currently evaluating whether this may represent a valid model for studying the TH gene expression related to the different HUMTH01 allele in vivo in healthy individuals, as well as in bipolar and schizophrenic patients.

The authors are planning the utilization of this model and other TH-expressing cell lines in combination with new genomic tools, such as the DNA decoy *(101,102)*, RNA interference *(103–105)*, and DNA microarray *(106)* methods. This will allow for the dissection of the mechanisms underlying the quantitative expression of the TH gene and, thus, may constitute a paradigmatic example of the approaches needed for the functional characterization of several genes implicated in normal and pathological complex genetic traits. Finally, the identification of the different components participating in the functional role of the HUMTH01 microsatellite may yield new therapeutic targets for

psychiatric diseases, such as bipolar disorder and schizophrenia.

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