increased, and body mass was lower when squirrels were feeding on these items as compared with feeding on seed (26, 27). Lastly, food supplementation experiments of American reds have failed to produce increases in litter size or a second litter, providing strong evidence that the increased reproductive investment observed in our study cannot be triggered by increased energy availability alone (17, 28, 29). We hypothesize that, rather than following a resource-tracking strategy where reproductive investment is determined by current resource amounts, reproductive rates are driven by future fitness payoffs. During years of low seed production, competition among juveniles for available resources is intense, and, although litter augmentation experiments in American reds show that females are capable of supporting larger litters (30), they refrain from doing so because offspring recruitment is low (30). However, when mast years occur, competition among juveniles is reduced, and females produce more offspring, which successfully recruit into the population (31, 32). Further, the increased production of young by females in mast years does not come with any obvious cost to the female, because overwinter survival is not reduced after years of increased reproductive investment (for American reds, offspring production in the previous year versus proportion of adult females surviving to spring has slope = 0.023 ± 0.034, \( t_{15} = -0.7 \), and \( P = 0.51 \); for Eurasian reds, proportion of estrous females in the previous year versus adult female survival to spring has slope = 0.37 ± 0.27, \( t_{15} = 1.4 \), and \( P = 0.19 \).

If mastaging has evolved as a swamp-and-starve adaptation against seed predation, then anticipatory reproduction and population growth represent a potent counteradaptation by the predators. Given that increased reproductive output in these systems coincides with low current but high future resources, our results suggest that reproductive investment in these systems is more responsive to future fitness prospects than present energetic constraints. The evolution of seed mastaging in trees is also driven by the survival prospects for progeny rather than simple resource tracking, suggesting an intriguing parallel in reproductive strategies of trees and the predators that consume their seed.

References and Notes
17. Materials and methods are available on Science Online.
33. We thank J. Herbers, C. Aumann, J. LaMontagne, M. Andruskiew, J. Lane, R. Boonstra, K. McCann, B. Thomas, Kluane Red Squirrel Project 2005 annual meeting participants, and three anonymous reviewers for very useful comments. We are indebted to the many field workers who helped collect the data and to A. Sykes and E. Anderson for management of the long-term data. Financed by Natural Sciences and Engineering Research Council of Canada, NSF (American reds), a Concerted Action of the Belgian Ministry of Education, and a Training and Mobility of Researchers grant from the Commission of the European Union (Eurasian reds). This is publication 30 of the Kluane Red Squirrel Project.

Supporting Online Material
www.sciencemag.org/cgi/content/full/314/5807/1928/DC1
Materials and Methods
SOM Text
Fig. S1
Table S1
References
25 September 2006; accepted 15 November 2006
10.1126/science.1135520

Human Catechol-O-Methyltransferase Haplotypes Modulate Protein Expression by Altering mRNA Secondary Structure

A. G. Nackley, 1 S. A. Shabalina, 2 I. E. Tchivileva, 1 K. Satterfield, 1 O. Korchynskyi, 3 S. S. Makarov, 4 W. Maixner, 1 L. Diatchenko 1*

Catechol-O-methyltransferase (COMT) is a key regulator of pain perception, cognitive function, and affective mood. Three common haplotypes of the human COMT gene, divergent in two synonymous and one non synonymous position, code for differences in COMT enzymatic activity and are associated with pain sensitivity. Haplotypes divergent in synonymous changes exhibited the largest difference in COMT enzymatic activity, due to a reduced amount of translated protein. The major COMT haplotypes varied with respect to messenger RNA local stem-loop structures, such that the most stable structure was associated with the lowest protein levels and enzymatic activity. Site-directed mutagenesis that eliminated the stable structure restored the amount of translated protein. These data highlight the functional significance of synonymous variations and suggest the importance of haplotypes over single-nucleotide polymorphisms for analysis of genetic variations.

The ability to predict the downstream effects of genetic variation is critically important for understanding both the evolution of the genome and the molecular basis of human disease. The effects of non synonymous polymorphisms have been widely characterized; because these variations directly influence protein function, they are relatively easy to study statistically and experimentally (1). However, characterizing polymorphisms located in regulatory regions, which are much more common, has proved to be problematic (2). Here, we focus on the mechanism whereby polymorphisms of the catechol-O-methyltransferase (COMT) gene regulate gene expression.

COMT is an enzyme responsible for degrading catecholamines and thus represents a critical component of homeostasis maintenance (3). The human COMT gene encodes two distinct proteins: soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) through the use of alternative translation initia-
tion sites and promoters (3). Recently, COMT has been implicated in the modulation of persistent pain (4–7). Our group demonstrated that three common haplotypes of the human COMT gene are associated with pain sensitivity and the likelihood of developing temporomandibular joint disorder (TMJD), a common chronic musculoskeletal pain condition (4). Three major haplotypes are formed by four single-nucleotide polymorphisms (SNPs): one located in the S-COMT promoter region (A/G; rs6269) and three in the S- and MB-COMT coding regions at codons his69his (C/T; rs4633), leu156leu (C/G; rs4818), and val158met (A/G; rs4680) (Fig. 1A). On the basis of subjects’ pain responsiveness, haplotypes were designated as low (LPS; GCGG), average (APS; ATCA), or high (HPS; ACCG) pain sensitive. Individuals carrying HPS/APS or APS/APS haplotypes were nearly 2.5 times as likely to develop TMJD. Previous data further suggest that COMT haplotypes code for differences in COMT enzymatic activity (4); however, the molecular mechanisms involved have remained unknown.

A common SNP in codon 158 (val158met) has been associated with pain ratings and μ-opioid system responses (7) as well as addiction, cognition, and common affective disorders (3, 8–10). The substitution of valine (Val) by methionine (Met) results in reduced thermostability and activity of the enzyme (3). It is generally accepted that val158met is the main source of individual variation in human COMT activity; numerous studies have identified associations between the low-activity met allele and several complex phenotypes (3, 8, 10). However, observed associations between these conditions and the met allele are often modest and occasionally inconsistent (3). This suggests that additional SNPs in the COMT gene modulate COMT activity. Indeed, we found that haplotype rather than an individual SNP better accounts for variability in pain sensitivity (4). The HPS and LPS haplotypes that both code for the stable val158 variant were associated with the two extreme-pain phenotypes; thus, the effect of haplotype on pain sensitivity in our study cannot be explained by the sum of the effects of functional SNPs. Instead, the val158met SNP interacts with other SNPs to determine phenotype. Because variation in the S-COMT promoter region does not contribute to pain phenotype (Fig. 1A), we suggest that the rate of mRNA degradation or protein synthesis is affected by the structural properties of the haplotypes, such as haplotype-specific mRNA secondary structure.

Previous reports have shown that polymorphic alleles can markedly affect mRNA secondary structure (11, 12), which can then have functional consequences on the rate of mRNA degradation (11, 13). It is also plausible that polymorphic alleles directly modulate protein translation through alterations in mRNA secondary structure, because protein translation efficiency is affected by mRNA secondary structure (14–16). To test these possibilities, we evaluated the affect of LPS, APS, and HPS haplotypes on the stability of the corresponding mRNA secondary structures (17).

Secondary structures of the full-length LPS, APS, and HPS mRNA transcripts were predicted by means of the RNA Mfold (18, 19) and Afold (20) programs. The mRNA folding analyses demonstrated that the major COMT haplotypes differ with respect to mRNA secondary structure. The LPS haplotype codes for the shortest, least stable local stem-loop structure, and the HPS haplotype codes for the longest, most stable local stem-loop structure in the val158 region for both S-COMT and MB-COMT (Fig. 1B and fig. S1A). Additional evidence supporting predicted RNA folding structures was obtained by generating consensus RNA secondary structures based on comparative analysis of COMT sequences from eight mammalian species (fig. S2). The consensus RNA folding structures were LPS-like and did not contain highly stable local stem-loop structures analogous to the human HPS-like form. Thus, substantial deviation from consensus structure, as observed for the HPS haplotype, should have notable functional consequences. Additional studies were conducted to test this molecular modeling.

We constructed full-length S- and MB-COMT cDNA clones in mammalian expression vectors that differed only in three nucleotides corresponding to the LPS, APS, and HPS haplotypes (17, 21). Rat adrenal (PC-12) cells were transiently transfected with each of these constructs. COMT enzymatic activity, protein expression, and mRNA abundance were measured. Relative to the LPS haplotype, the HPS haplotype showed a 25- and 18-fold reduction in enzymatic activity for S- and MB-COMT constructs, respectively (Fig. 1C and fig. S1B). The HPS haplotype also exhibited marked reductions in S- and MB-COMT protein expression (Fig. 1D and fig. S1C). The APS haplotype displayed a moderate 2.5- and 3-fold reduction in enzymatic activity for S- and MB-COMT constructs, respectively, while pro-

---

**Fig. 1.** Common haplotypes of the human COMT gene differ with respect to mRNA secondary structure and enzymatic activity. (A) A schematic diagram illustrates COMT genomic organization and SNP composition for the three haplotypes. Percent frequency of each haplotype in a cohort of healthy Caucasian females, and percent independent SNP contribution to pain sensitivity, are indicated. (B) The local stem-loop structures associated with each of the three haplotypes are shown. Relative to the LPS and APS haplotypes, the HPS local stem-loop structure had a higher folding potential. (C and D) The LPS haplotype exhibited the highest, while the HPS haplotype exhibited the lowest enzymatic activity and protein levels in cells expressing COMT. ***P < 0.001, ≠ LPS, ***P < 0.001, ≠ APS.
tein expression levels did not differ. The moderate reduction in enzymatic activity produced by the APS haplotype is most likely due to the previously reported decrease in protein thermostability coded by the met<sup>158</sup> allele (3). These data illustrate that the reduced enzymatic activity corresponding to the HPS haplotype is paralleled by reduced protein levels, an effect that could be mediated by local mRNA secondary structure at the level of protein synthesis and/or mRNA degradation. Because total RNA abundance and RNA degradation rates did not parallel COMT protein levels (fig. S2), differences in protein translation efficiency likely results from differences in the local secondary structure of corresponding mRNAs.

To directly assess this hypothesis, we performed site-directed mutagenesis (17). The stable stem-loop structure of S- and MB-COMT mRNA corresponding to the HPS haplotype is supported by base pairs between several critical nucleotides, including 403C and 479G in S-COMT and 625C and 701G in MB-COMT (fig. 2A and fig. S3A). Mutation of 403C to G in S-COMT or 625C to G in MB-COMT destroys the stable stem-loop structure and converts it into a LPS haplotype-like structure (HPS Lsm). Double mutation of mRNA in position 403C to G and 479G to C in S-COMT or 625C to G and 701G to C in MB-COMT reconstructs the original long stem-loop structure (HPS dm). The single- and double-nucleotide HPS mutants (HPS Lsm and HPS dm, respectively) were transiently transfected to PC-12 cells. As predicted by the mRNA secondary-structure folding analyses, the HPS Lsm exhibited increased COMT enzymatic activity and protein levels equivalent to those of the LPS haplotype, whereas the HPS dm exhibited reduced enzymatic activity and protein levels equivalent to those of the original HPS haplotype (Fig. 2, B and C; fig. S3, B and C). These data rule out the involvement of RNA sequence recognition motifs or codon usage in the regulation of translation. In contrast to the HPS haplotype, protein levels did not parallel COMT enzymatic activity for the APS haplotype and site-directed mutagenesis confirmed that the met<sup>158</sup> allele, not a more stable mRNA secondary structure, drives the reduced enzymatic activity observed for the APS haplotype (fig. S4). This difference is moderate relative to the mRNA structure–dependent difference coded by LPS and HPS haplotypes. These results were verified by an alternate approach of modifying mRNA secondary structure (fig. S5).

![Fig. 2. Site-directed mutagenesis that destroys the stable stem-loop structure corresponding to the HPS haplotype restores COMT enzymatic activity and protein expression.](image)

**A** The mRNA structure corresponding to the HPS haplotype was converted to an LPS haplotype-like structure (HPS Lsm) by single mutation of 403C to G. The original HPS haplotype structure (HPS dm) was restored by double mutation of interacting nucleotides 403C to G and 479G to C. (B and C) The HPS Lsm exhibited COMT enzymatic activity and protein levels equivalent to those of the LPS haplotype, whereas the HPS dm exhibited reduced enzymatic activity. ***P < 0.001, ≠ LPS.**

Our data have very broad evolutionary and medical implications for the analysis of variants common in the human population. The fact that alterations in mRNA secondary structure resulting from synonymous changes have such a pronounced effect on the level of protein expression emphasizes the critical role of synonymous nucleotide positions in maintaining mRNA secondary structure and suggests that the mRNA secondary structure, rather than independent nucleotides in the synonymous positions, should undergo substantial selective pressure (22). Furthermore, our data stress the importance of synonymous SNPs as potential functional variants in the area of human medical genetics. Although nonsynonymous SNPs are believed to have the strongest impact on variation in gene function, our data clearly demonstrate that haploptic variants of common synonymous SNPs can have stronger effects on gene function than nonsynonymous variations and play an important role in disease onset and progression.

**References and Notes**

17. Materials and methods are available as supporting material on Science Online.
21. Sequence accession numbers: S-COMT clones BG290167, CA489448, and BF037202 represent LPS, APS, and HPS haplotypes, respectively. Clones corresponding to all three haplotypes and including the transcriptional start site were constructed by use of the unique restriction enzyme Bsp MI. The gene coding region containing all three SNPs was cut and inserted into the plasmids through use of the S-COMT (BG290167) and MB-COMT (B835796) clones containing the entire COMT 5′ and 3′ and 8′ ends as a backbone vector.
Lineages of Acidophilic Archaea Revealed by Community Genomic Analysis


Novel, low-abundance microbial species can be easily overlooked in standard polymerase chain reaction (PCR)–based surveys. We used community genomic data obtained without PCR or cultivation to reconstruct DNA fragments bearing unusual 16S ribosomal RNA (rRNA) and protein-coding genes from organisms belonging to novel archaeal lineages. The organisms are minor components of all biofilms growing in pH 0.5 to 1.5 solutions within the Richmond Mine, California. Probes specific for 16S rRNA showed that the fraction less than 0.45 micrometers in diameter is dominated by these organisms. Transmission electron microscope images revealed that the cells are pleomorphic with unusual folded membrane protrusions and have apparent volumes of ~0.006 cubic micrometer.

Our understanding of the variety of microorganisms that populate natural environments was advanced by the development of polymerase chain reaction (PCR)–based, cultivation-independent methods that target one or a small number of genes (1–3). Genomic analyses of DNA sequence fragments derived from multispecies consortia (4–6) and whole environments (7–9) have provided new information about diversity and metabolic potential. However, PCR-based methods have limited ability to detect organisms whose genes are significantly divergent relative to gene sequences in databases, and most cultivation-independent genomic sequencing approaches are relatively insensitive to organisms that occur at low abundance. Consequently, it is likely that low-abundance microorganisms distantly related to known species will be undetected members of natural consortia, even in low complexity systems such as acid mine drainage (AMD) (10).

An important way in which microorganisms affect geochemical cycles is by accelerating the dissolution of minerals. For example, microorganisms can derive metabolic energy by oxidizing iron released by the dissolution of pyrite (FeS₂). The ferric iron by-product promotes further pyrite dissolution, leading to AMD generation. AMD solutions forming underground in the Richmond Mine at Iron Mountain, California, are warm (30° to 59°C), acidic (pH ~0.5 to 1.5), metal-rich [submolar Fe²⁺ and micromolar As and Cu ([11]) and host active microbial communities. Extensive cultivation-independent sequence analysis of functional and rRNA genes ([11, 12]) revealed that biofilms contain a significant number of Archaea, but the diversity reported to date has been limited to the order Thermoplasmatales ([10]). Current models for AMD generation thus include only these species.

The genomes of the five dominant members of one biofilm community from the “5-way” region of the Richmond Mine (Fig. S1) were largely reconstructed through the assembly of 76 Mb of shotgun genomic sequence (4). Previously unreported is a genome fragment that encodes part of the 16S rRNA gene of a novel archaean lineage: Archaeal Richmond Mine Acidophilic Nanoorganism (ARMAN-1). Using an expanded data set that now comprises more than 100 Mb of genomic sequence, we reconstructed a contiguous 4.2-kb fragment adjacent to this gene. A second 13.2-kb genome fragment encoding a 16S rRNA gene from an organism that is related to ARMAN-1 (ARMAN-2) was reconstructed from 117 Mb of community genomic sequence derived from a biofilm from the A drift (Fig. S1). Within the data sets from each site, results to date indicate that each ARMAN population is near-clonal.

Comparison of the ARMAN-1 and -2 DNA fragments revealed some gene rearrangements, insertions, and deletions (Fig. 1). Genes present in both organisms encode putative inorganic pyrophosphatases, a transcription regulator, and a gene shown to be an arsenate reductase (13). Comparative analysis of these genes with sequences in the public databases consistently

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Comparison of syntenous genomic regions of ARMAN-1 [from the “5-way” (CG) community (4)] and ARMAN-2 [from the UBA community]. Orthologs and their protein identity are indicated by the red bands. The percentage similarity for the 16S rRNA gene sequences is also shown. Numbers at ends indicate length (number of nucleotides); predicted open reading frames for hypothetical proteins are indicated by boxes.