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Candidate Genes for Alcohol Dependence: Animal Studies

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ALCOHOL DEPENDENCE IS a complex disorder with environmental and genetic components. Its strong heritability component has been demonstrated in family, twin, and adoption studies (Reich et al., 1999). Alcohol dependence is a polygenic disorder, and segregation analyses suggest that a major gene is not likely to be operative in this disorder (Enoch and Goldman, 1999; Goldman, 1993). Thus, any single gene that contributes to the vulnerability of alcohol dependence might produce a relatively small effect.

Complex traits such as alcohol dependence are well suited for genetic analyses using a candidate gene approach. Whereas linkage analyses are the method of choice for rare single gene effects, candidate gene studies are especially powerful in detecting genes for which the risk associated with any given candidate gene is relatively small (Risch and Merikangas, 1996). Candidate genes are usually identified by two basic approaches: (1) positional cloning, the isolation of a gene on the basis of its chromosomal location, regardless of its biochemical function, and (2) functional identification of genes such as in animal models. Whereas linkage results may underscore the potential relevance of a candidate gene encoded in a region of interest, a selection of candidate genes based exclusively on results of linkage analyses and positional cloning experiments may not be sufficiently sensitive. Therefore, functional identification of genes that contribute to the risk of alcoholism and are based on biochemical or behavioral studies in different model organisms is an important approach.

Recent advances in molecular genetics have provided methods and tools to exploit the information on candidate genes generated by basic science studies in association studies of genetic variations in patients with alcohol dependence. Whereas earlier, analyses of genetic variations had been performed using few Single Nucleotide Polymorphisms (SNPs) in a limited number of genes, now, as a result of the progress of the human genome project, systematic mutational analyses and detection of all SNPs in any given candidate gene are possible.

In this review, we focus on animal models that have led to the identification of candidate genes for alcohol dependence and describe recent methods for identification and genotyping of SNPs. For an overview on the most promising candidate genes currently identified and analyzed in humans, please refer to the review in this issue by Dick and Foroud.

IDENTIFICATION OF CANDIDATE GENES IN ANIMAL STUDIES

The appropriate choice of candidate genes is dependent on our knowledge of the pathophysiology of alcohol dependence and the gene products and biochemical mechanisms underlying this disorder. Although many of the receptors, signaling pathways, gene expression patterns, and cellular communication that determine the biology of alcohol dependence still remain obscure, recent advances using animal models have provided new candidate genes.

Candidate Genes From Alcohol Sensitivity Studies in Drosophila

Drosophila melanogaster can metabolize alcohol efficiently, because fermenting fruits, which often contain alcohol levels of 3% or more, are part of the natural habitat of fruit flies (Geer et al., 1993). When exposed to alcohol, *Drosophila* displays behaviors comparable to mammals and humans, including decreased locomotion and loss of postural control. Both behaviors can be quantified experimentally and serve as a measure of alcohol sensitivity (Heberlein, 2000). Identification of mutations that alter alcohol sensitivity has been performed by screening of flies with randomly inactivated genes using chemical mutagenesis or randomly integrating transposable elements (Geer et al., 1993). These experiments have led to the identification of genes that modulate sensitivity to alcohol by regulating the

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adenosine 3':5'-cyclic monophosphate (cAMP) pathway. cAMP activates protein kinase A (PKA), which subsequently phosphorylates nearby substrates, and with prolonged activation it translocates to the nucleus and regulates gene expression (Yao et al., 2002). cAMP production is controlled in part by adenylate cyclase (AC). While mutations of the gene "amnesiac," the *Drosophila* homologue of the human adenylate cyclase gene (Feany and Quinn, 1995), lead to enhanced alcohol sensitivity (Moore et al., 1998), mutations in cAMP-regulated protein-kinase (PKA-RII) decreased alcohol sensitivity (Park et al., 2000).

Similar to *Drosophila*, studies in PKA-RII knock-out mice have revealed reduced alcohol sensitivity (Thiele et al., 2000), demonstrating the evolutionary conservation of molecular mechanisms of drug responses in *Drosophila* and mice (Heberlein, 2000). The finding that alcohol responsive genes are highly conserved throughout evolution is supported by studies on clock genes. On the basis of observations that the circadian clock gene period (Per) can influence behavioral responses to cocaine in *Drosophila* (Andretic et al., 1999), Abarca et al. (2002) showed in knock-out mice that the homologue of the *Drosophila* Per gene, mPer1, modulates cocaine sensitization and reward as well as alcohol sensitivity (Abarca C, Albrecht U, Spanagel R, Alcohol sensitivity is regulated by clock genes, Submitted). Although unconditioned drug effects such as alcohol sensitivity can be studied in *Drosophila*, this species is limited in its use for operant conditioning and self-administration studies. Mouse mutants, however, are thought to be a valuable tool to identify genes involved in the regulation of voluntary alcohol consumption and alcohol reinforcement processes.

Candidate Genes From Alcohol Self-Administration Studies in Mouse Mutants

With the use of a two-bottle free-choice paradigm, knock-out mouse studies have generated a wide variety of candidate genes associated with voluntary alcohol consumption and alcohol preference. Several candidate genes revealed by knock-out mice, however, were previously identified by pharmacological studies; therefore, their role in voluntary alcohol consumption and preference was only further confirmed by the knock-out model. Examples for this are the dopamine D1 and D2 receptor knock-out models: Behavioral pharmacological experiments with selective D1 and D2 dopamine receptor antagonists demonstrated reduced alcohol intake in rodents (Hodge et al., 1997), a finding that was then largely confirmed by D1 and D2 dopamine receptor knock-outs that showed less alcohol intake and preference than their wild-type controls (El-Ghundi et al., 1998; Phillips et al., 1998). However, discrepancies between pharmacological data and data derived from knock-out animals have also been reported. Thus, recently a new role of the neuronal nitric oxide synthase (nNOS) gene in the regulation of alcohol drinking behavior was described. nNOS is an intermediate gene in the glutamatergic signal transduction pathway that leads to activation of CREB (cAMP response binding element) (Kiss and Vizi, 2001; Sasaki et al., 2000). nNOS knock-outs consumed 6-fold more alcohol from highly concentrated alcohol solutions than wild-type mice (Spanagel et al., 2002). These findings contrast with previous reports that showed that nonselective NOS inhibitors decrease alcohol consumption (Calapai et al., 1996; Lallemand and De Witte, 1997; Rezvani et al., 1995). However, because alcohol consumption was suppressed in wild-type as well as in nNOS $-/-$ mice by the NOS inhibitor L-NAME (nitro-L-arginine methyl ester), it is concluded that the effect of nonselective NOS inhibitors on alcohol drinking is not mediated by nNOS (Spanagel et al., 2002). This study exemplifies that pharmacological examinations with unselective antagonists can be misleading and that knock-out models provide a high degree of selectivity to study the function of a particular gene.

Apart from studying genes that modify alcohol drinking behavior in a direct way, mouse knock-out models can be used to analyze the interaction of alcohol drinking and response to environmental factors, such as stress. Mice that lack a functional corticotropin-releasing hormone receptor 1 (CRHR1) receptor did not differ from wild-type control animals in alcohol consumption and preference when submitted to a two-bottle free-choice procedure. However, after repeated stress, CRHR1 knock-outs showed enhanced alcohol intake. This stress-induced alcohol drinking behavior appeared with a delay and persisted throughout life (Sillaber et al., 2002). Alterations in the CRHR1 receptor gene may therefore constitute a genetic risk factor for stress-induced alcohol drinking and alcoholism. To our knowledge, this is the first demonstration that a gene deletion leads to a "silent phenotype" in terms of alcohol drinking. However, with the interplay of environmental stimuli such as stress, a clear phenotype emerges, supporting the notion that gene–environment interactions also have to be studied to achieve conclusions about a gene function in alcohol drinking behavior.

Whereas knock-out mice have provided confirmatory evidence for the role of candidate genes in alcohol preference, one limitation of this model has been the disregard of developmental consequences as well as lack of tissue specificity of a gene knocked out nonselectively in the embryonal stem cell. Furthermore, elimination of a gene in embryonic stem cells can lead to compensatory mechanisms masking the effect of the candidate gene on the phenotype analyzed. Now, this problem can be overcome in part by using inducible transgenic/knock-out animals with site-specific promoters. One approach is to construct a tetracycline-sensitive activator (Mansuy and Bujard, 2000), which can induce a site-specific promoter, such as the CaMKIIα promoter for hippocampal and forebrain specific gene expression. The promoter activates transcription of the transgene carrying additional copies of the target gene.

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To obtain a knock-out mouse, cre-recombinase can be introduced under the control of a site-specific, inducible promoter. A different mouse strain in which lox P-sites flank the gene to be eliminated is simultaneously generated. Upon crossing of the two mouse strains, offspring that carry both the cre-recombinase and a gene of interest flanked by lox P-sites will be created. After activation of the cre-recombinase promoter construct with tetracycline, the cre-recombinase will eliminate the target gene and a site-specific knock-out mouse is generated (for review see, Kuhn and Torres, 2002). Because this method is methodically complex and time consuming, its application is limited to a few specialized laboratories and thus is not amenable to routine assessment of gene function. An example for a tissue-specific inactivation of a gene was recently presented by Cowen et al. (2001). As genetic inactivation of the glucocorticoid receptor (GR) causes lethality at birth, a mouse strain with a nervous system-specific knock-out (Tronche et al., 1999) was studied in a two-bottle, free-choice procedure. Central nervous system GR knock-out mice voluntarily drank more ethanol than control mice. This study demonstrates that the activation of GRs in the brain is involved in the regulation of alcohol self-administration and that alterations in the GR gene may constitute a genetic risk factor for the vulnerability of alcohol dependence.

Although inbred mouse strains with different behavioral responses to ethanol have been identified (Belknap et al., 1993), epistatic interactions in knock-out models have rarely been systematically addressed. However, in the case of the protein kinase C (PKC) knock-out mouse (Harris et al., 1995), it has been shown that the observed decreased alcohol sensitivity and tolerance is dependent on the genetic background of the animal. Whereas null mutants of the parental strains C57BL/6J and 129/SvJ developed tolerance, null mutants from the C57BL/6J × 129/SvJ F2 generation did not exhibit tolerance. Epistatic interactions between the targeted γ-PKC null mutation and multiple genes from the parental strain were thought to account for the lack of tolerance observed in null mutants from this mixed background (Bowers et al., 1999). This example highlights the oligogenic nature of alcohol-related phenotypes and points toward the complex interaction of genes involved in regulating alcohol drinking behavior.

Another limitation that has to be considered in working with knock-out animals is that the absence of a specific gene can interrupt an entire signal transduction cascade; hence, any gene mutation upstream or downstream of the knock-out may be pathophysiologically causative in influencing alcohol drinking behavior. Ethanol-induced glutamatergic neurotransmission is an example for this phenomenon. NMDA-mediated signaling has been shown to influence pathophysiological mechanisms central to the development of alcohol dependence, including tolerance, withdrawal symptoms, craving, and ethanol-related neurotoxicity (Tsai et al., 1995). NMDA receptors are regulated by PKC (Benquet et al., 2002) and the src-like protein tyrosine kinase (PTK) fyn (Cheung and Gurd, 2001) and induce activation of PKA as well as nNOS (Sheng and Kim, 2002). PKC (Bowers et al., 1999; Harris et al., 1995), PTK fyn (Miyakawa et al., 1997), PKA (Thiele et al., 2000), and nNOS knock-out mice (Spanagel et al., 2002) showed altered sensitivity to the effects of ethanol. This may be interpreted as indicating a general involvement of NMDA-mediated glutamatergic signal transduction in the regulation of alcohol sensitivity, which can be manipulated at different levels causing a similar phenotype. Furthermore, knocking out a specific gene does not usually correspond to the effect of genes on the pathophysiology of frequent, oligogenic human disorders, where a gradual change in gene activity is likely.

Therefore, to reflect the complexity of biochemical mechanisms involved in regulating alcohol drinking behavior in the design of human genetic studies, it may be reasonable to systematically map an entire signaling cascade by performing mutation analyses and association studies of the genes encoding key signaling molecules. These key molecules can be identified on the basis of gene expression profiling studies in the particular knock-out and wild-type animals of different strains. Alternatively, candidate genes from knock-out studies can be compared with results from mouse quantitative trait loci (QTL) analyses.

Candidate Genes From Mouse QTL Analyses

A complementary approach for identification of candidate genes that is less prone to problems with respect to total loss of gene activity is mouse QTL analyses. As in human linkage studies, these approaches to identify alcohol-related genes are based on their location in the genome. These studies use genetic animal models, such as inbred strains or animals that have been selectively bred for a specific phenotype. The models can be analyzed for linkage of genetic markers and phenotypes such as ethanol withdrawal severity, ethanol preference, ethanol sensitivity, and tolerance (Phillips and Crabbe, 1991). Because phenotypes related to alcohol dependence are based on the interaction of multiple genes, single candidate genes have a relatively small effect on individual risk. Therefore, the population distribution of animals scoring high or low on the mapped loci usually is quantitatively distributed (for review, see Crabbe, 2002). Linked QTLs encompass a chromosomal region that might contain hundreds of genes. To reduce the confidence interval of a QTL on chromosome 4 that was linked to high alcohol withdrawal severity (Buck et al., 1997), Fehr et al. (2002) generated congenic strains by introducing a small region of this QTL by repeated back-crossing from an inbred strain with high alcohol withdrawal severity into an inbred strain not carrying this phenotype. With the use of this approach, a congenic strain containing fewer than 20 genes in the region of interest was generated. Mutation analysis of these genes revealed a multiple PDZ-

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domain zinc finger protein gene, Mpdz. The Mpdz gene carried functional genetic variations that predicted three different Mpdz protein variants and that were associated with altered withdrawal characteristics in different congenic strains. This work is an example for QTL-based identification of candidate genes. In the future, this approach will be greatly facilitated by advances in mouse genomics, such as the publication of a physical map of the mouse genome (Gregory et al., 2002), the ongoing sequencing of the mouse genome (http://www.ncbi.nlm.nih.gov/genome/seq/MmHome.html), and the complete sequencing of the mouse transcriptome (Okazaki et al., 2002).

On the basis of evidence for a role for cAMP signaling in acute and chronic effects of ethanol (see above and review by Tabakoff et al., 2001) Kirstein and Tabakoff (2001) analyzed several inbred strains of mice and observed a genetic correlation of alcohol sensitivity (initial ataxic sensitivity as assessed by the stationary dowel) and cAMP signaling as assessed by measurement of basal accumulation as well as isoproterenol- and forskolin-stimulated accumulation of cAMP in the cerebellum and the basal ganglia. In a follow-up study, provisional QTL were identified for initial sensitivity to ethanol, acute functional tolerance, and cAMP signaling on different chromosomes (Kirstein et al., 2002).

A combined approach using linkage studies and functional genetics with knock-out animals proved successful in providing new insights into the role of neuropeptide receptors for alcohol drinking behavior: In 1998, a QTL on rat chromosome 4 was identified through selective breeding of alcohol preferring (P) and alcohol nonpreferring (NP) rat lines (Carr et al., 1998). This locus accounted for 11% of the total phenotypic variability and approximately one third of the total genetic variability. The gene for NPY was located at the peak of the linkage region, stimulating interest in NPY as a candidate gene for alcohol dependence. It was subsequently demonstrated that NPY-deficient mice have increased consumption of ethanol, as compared with wild-type mice, and are less sensitive to the hypnotic effects of ethanol (Thiele et al., 1998). More recently, it was shown that knock-out mice that lack the NPY Y1 receptor have increased consumption of ethanol, suggesting that the involvement of NPY may be via Y1 receptor signaling (Thiele et al., 2002). In contrast, transgenic mice with overexpression of NPY show decreased levels of alcohol consumption and increased sensitivity to the effects of ethanol. NPY immunoreactivity is reduced in the amygdala of both P rats and an independent line of high alcohol drinking (HAD) rats, as compared with their lower drinking counterparts (Hwang et al., 1999).

Candidate Genes From Rat Models for Alcohol Dependence

The paradigms investigated in mouse mutants and QTL analyses have identified a number of alcohol-related phenotypes that are, nevertheless, limited with respect to the complex behavioral aspects of human alcohol dependence. Therefore, over the past 15 years, researchers have developed new animal models that mimic different aspects of human alcohol dependence, such as relapse and loss of control over drinking. These models include the reinstatement model and the alcohol deprivation model (for review, see Spanagel, 2000).

The reinstatement model is the first choice for the measurement of relapse behavior (Stewart and de Wit, 1987). In this paradigm, the animal is trained to self-administer a drug via lever pressing and is then subjected to extinction—that is, it is tested under conditions of nonreinforcement until operant responding seems to be extinguished. When the animal reaches some criterion of unresponsiveness, various stimuli are presented. A stimulus is said to reinstate the drug-seeking behavior if it causes renewed responding, i.e., lever pressing. At least three stimuli can reinstate responding: (1) Chiamulera et al. (1995) showed that a small quantity of ethanol is able to reinstate previously extinguished alcohol-seeking behavior. This finding is consistent with the widely reported description of the "first-drink" phenomenon: ingestion of a small quantity of alcohol may lead to relapse in abstinent alcoholics (Ludwig et al., 1974). (2) A second stimuli is stress (e.g., footshock stress) that can reinstate alcohol-seeking behavior (Lê et al., 1998). (3) Conditioned stimuli previously associated with alcohol intake can also reinstate extinguished alcohol-seeking behavior (Katner et al., 1999).

In another animal model for relapse—the alcohol deprivation model—a compulsive uncontrolled drug-taking behavior can be observed (Spanagel and Hölter, 1999). The experimental procedure is as follows: Rats receive food and tap water and alcohol solutions (5, 10, and 20% v/v) ad libitum in four bottles per cage. After 2 months of continuous alcohol access, rats are deprived of alcohol for several days. After this deprivation phase, all alcohol solutions are presented again. This procedure is repeated regularly for the following year. The re-presentation of the alcohol solutions after a deprivation phase leads to a pronounced transient rise in alcohol intake and preference that is called the alcohol deprivation effect (which can be viewed as relapse-like drinking behavior). The alcohol deprivation effect changes its characteristics with repeated deprivation phases. In particular, long-term alcohol-drinking rats that had repeated deprivation phases subsequently consume large amounts of highly concentrated alcohol solutions and show changes in the diurnal rhythm of drinking activity (Spanagel and Hölter, 1999). Furthermore, the alcohol deprivation effect is prolonged and enhanced in P rat lines after repeated deprivation phases (Rodd-Henricks et al., 2000, 2001). Long-term alcohol experienced rats that underwent repeated alcohol deprivation phases also exhibited tolerance, physical as well as psychic signs of withdrawal, and stress-induced drinking (Hölter et al., 1998; 2000; Spanagel and Hölter, 2000). In conclusion, this model reflects some of the diagnostic criteria for alcohol abuse and de-

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pendence given in the DSM-IV and can, therefore, be considered as an animal model of alcohol dependence.

The use of appropriate animal models is a key component in functional studies of genes involved in normal and pathologic brain function. The reinstatement model and the alcohol deprivation model in rats described here reflect key aspects of human alcoholism and are, therefore, ideal tools to identify candidate genes via gene expression profiling. A promising approach for gene expression profiling is the DNA-microarray technology, whereby DNA fragments specific for selected genes are spotted on a chip made of glass, silicone, or nitrocellulose. This chip is hybridized with cDNA that has been derived from reverse transcribed RNA from specific regions of brains from alcohol-dependent versus nondependent animals. The readout provides a semiquantitative analysis of transcriptional activity of genes induced or inhibited after alcohol drinking behavior (for an extensive review, see Heller, 2002).

Published studies have used microarrays to analyze the transcriptional activation pattern after ethanol administration in rats (Rimondini et al., 2002), neuronal cell lines (Thibault et al., 2000), and humans (Lewohl et al., 2000; Mayfield et al., 2002). The studies in human frontal and motor cortices observed differential regulation of genes already implicated in alcohol dependence, such as NPY. They highlighted the transcriptional down-regulation of genes involved in protein trafficking and myelin-related genes. It is interesting that ethanol-induced transcriptional activation in human neuroblastoma cells as well as in human frontal and motor cortices led to differential regulation of genes involved in cAMP signaling. Although the genes identified were not identical, these results complement the findings of QTL studies discussed above, demonstrating a role of cAMP signaling in alcohol sensitivity.

Rimondini et al. (2002) applied the microarray technique in an experiment with Wistar rats, which was designed to detect persistent changes in gene expression in rats that voluntarily drank alcohol and had had a previous experience with repeated deprivation phases. Expression analysis of cingulate cortex and amygdala with microarray chips revealed a set of >30 differentially regulated transcripts in this model. These include members of pathways previously implicated in alcohol dependence (glutamatergic, endocannabinoid, and monoaminergic neurotransmission), as well as pathways not previously thought to be involved in this disorder. More specific, glutamate receptors and transporters were up-regulated in the cingulate cortex, supporting the hypothesis that functional changes in the glutamatergic system contribute to alcohol dependence (Spanagel and Bienkowski, 2002; Tsai et al., 1995).

FUNCTIONAL VALIDATION OF PUTATIVE CANDIDATE GENES IN ANIMAL STUDIES

Expression profiling data from rat models for alcohol dependence can be the first step toward new candidate genes. However, before this knowledge is transferred to an association study with alcoholic patients, functional validation of the putative candidate gene in the animal is warranted. A putative candidate gene can be considered as validated if a pharmacological or molecular manipulation of a gene that is overexpressed under conditions of high alcohol drinking and relapse behavior will lead to normalization or suppression of this particular behavior. Besides pharmacological validation with specific antagonists or antibodies, two novel molecular validation approaches can be used.

One approach is the use of transgenic rats. Unfortunately, to this day, only embryonic stem cells (ES) derived from mice strain 129 can be reintroduced reliably to the germline, thus allowing targeted gene ablation (knock-out). Therefore, much of the neurobiological research studying function of genes involved in pathologic processes originally performed using rats has been transferred to mice, but the differences in rat and mice biology limits the utility of mouse models. For example, it will be difficult to establish a reinstatement procedure in mice because of high motor activity and poor performance in goal-directed behaviors. Therefore, in studies involving complex disorders such as alcohol dependence, the genetic manipulation of already established rat models such as the reinstatement paradigm would be a real advantage. Although the knock-out technology is not readily available in rats, the generation of transgenic rats by pronuclear injection of DNA is technically possible.

Another approach is the use of gene or antisense oligonucleotide delivery in the adult rat. Recombinant viral vectors, which deliver genes in vivo, and antisense oligonucleotides, which can specifically block or destroy RNA transcribed from candidate genes, have already been used in alcohol research (Israel et al., 2002) and may provide suitable tools in the future. Recently, a technique that inhibits gene expression using RNA interference (RNAi) has been presented (Fire et al., 1998). RNAi is triggered by the presence of double-stranded RNA (dsRNA) in the cell and results in the rapid destruction of the mRNA containing identical or nearly identical sequences. It has recently been shown that RNAi can be induced in cultured mammalian neurons (Krichevsky and Kosik, 2002) and delivered for inhibition of gene expression in postnatal rodents (Lewis et al., 2002). Because of its potency and specificity, this method holds considerable promise, both for research applications such as candidate gene validation and for development of new drugs targeted against genes that cause alcohol dependence.

In summary, the identification of candidate genes on the basis of animal experiments should take into consideration results of genetically altered animals in simple models for alcohol sensitivity and alcohol preference as well as the analysis of animals that derive from models for alcohol dependence and undergo thorough gene profiling analysis. Whereas transgenic/knock-out studies provide information

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about the contribution of a single gene that may or may not be pathophysiologically causative, gene profiling will delineate additional candidates for pathophysiologically relevant genes that are functionally related to the gene under investigation. Candidate genes derived from this integrated approach can then be prioritized with consideration of the results of linkage analyses.

METHODS FOR IDENTIFICATION AND GENOTYPING OF SNPS

SNPs are believed to account for 90% of the individual genetic variations (Brookes, 1999). Functional SNPs lead to missense mutations and consequently to an alteration of transcriptional activity or a change in the amino acid sequence of the encoded molecule, thus possibly influencing protein function. Nonfunctional (or silent) SNPs presently serve as markers for a genetic variation in the vicinity of its chromosomal location. Silent SNPs, however, may also lead to altered gene transcription as a result of generation of stop codons or of influencing of transcriptional efficiency via mechanisms that are presently unclear.

In the laboratory, systematic identification of SNPs in candidate genes became feasible by the development of high-throughput methods, such as automated capillary sequencing and heteroduplex HPLC (Oefner and Underhill, 1998). Heteroduplex HPLC is based on the differential elution of homologous and heterologous double-stranded DNA, dependent on the presence of a mismatch as a result of an SNP. The differential elution can be visualized as changes in the peak pattern of the HPLC. This system has been commercialized as WAVE-DNA analysis system by Transgenomic, Inc. (San Jose, CA). Although this approach has higher throughput capabilities than direct DNA sequencing, the presence of a suspected SNP must be confirmed by sequencing analysis. Using a 16-capillary automated sequencer, ~90,000 nucleotides can be sequenced per 24 hr.

Direct DNA sequencing was the method of choice for the SNP consortium that has made the largest contribution to genome-wide identification of SNPs. This organization, which was founded in 1999 by 14 companies and 4 academic institutions, has identified and made publicly available more than 1.5 million SNPs (http://www.snp.cshl.org). Apart from the SNP consortium, other public databases exist, such as HG-base (http://hgbase.interactiva.de) (Brookes et al., 2000) or dbSNP (NCBI) (http://www.ncbi.nlm.nih.gov/SNP/). Most SNPs identified in public databases remain unverified, and there is a considerable percentage of erroneous SNPs as a result of—for example—sequencing errors. Therefore, unverified SNPs derived from public databanks need to be confirmed by a genotyping method and assessed for allele frequency. Recently, a public database publishing proven SNPs that have been verified in a Japanese population was made available (http://gdb.jst.go.jp/HOWDY). Public databases are regularly adding information as to population frequency or allele distribution. Identification of linkage disequilibrium patterns—a measure of chromosomal rearrangements—will further develop genotype analysis by providing the tools to select and combine a set of specific SNP markers (haplotypes) and analyze their association with disease phenotypes. The so-called "haplotype map" program is a coordinated international effort designed to identify a set of common SNPs that could be used to tag haplotypes spanning the human genome that occur with >5% frequency in major ethnic groups. On the basis of reports in the literature, it is anticipated that the number of SNPs required to genotype informative haplotypes is <10% of the frequent SNPs present (Patil et al., 2001). Linkage disequilibrium patterns have already been studied across several chromosomes (Ardlie et al., 2002). Within the next years, it seems likely that SNP markers will be available for haplotypes covering at least large portions of the genome for association-based genetic analyses of various diseases.

As the techniques to identify SNPs have improved, so have the methods to genotype known SNPs from candidate genes. Whereas in the past, genotyping was performed using gel-based methods, now non–gel-based technologies that allow high-throughput genotyping at a significantly reduced cost are evolving. These technologies, which detect SNPs in a sequence-specific way, are based on four general mechanisms for allelic discrimination: allele-specific hybridization, allele-specific nucleotide incorporation (primer extension), allele-specific oligonucleotide ligation, and allele-specific invasive cleavage. Allelic discrimination techniques can be combined with different detection mechanisms, examples of which are luminescence, fluorescence, fluorescence resonance energy transfer, fluorescence polarization, and mass spectrometry detection (for review, see Kwok, 2001). These technologies differ with respect to their adaptability and ease of assay development as well as their suitability for large sample sizes. In a research setting, in which many markers need to be genotyped in populations of variable size, such as genetics of alcohol dependence, assay development must be simple and the assay needs to be adaptable to both low and high numbers of samples. The initial cost of assay development as well as the instruments involved should be moderate, so as to be affordable for medium-sized laboratories.

Primer extension is a robust allelic discrimination method that is flexible and requires small amounts of primers or probes. Probe design and optimization of the assay method are straightforward. In our laboratory, we use an assay based on allele-specific primer extension coupled with fluorescence polarization detection. The polymerase chain reaction product containing the polymorphic site serves as template, and the 3' end of the primer extension probe is immediately adjacent to the allelic base. With the use of one of two dye-labeled terminators that contain the nucleotides specific for the genetic variation, a polymerase extends the 3' end of the primer, thus labeling the respec-

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tive allele. The primer extension reaction greatly increases the molecular weight of the dye-labeled ddNTP terminator, thus decreasing molecular rotation. Molecular rotation is measured by fluorescence polarization. Polarization values are inversely related to the speed of molecular rotation. This test retains the sensitivity and specificity of the primer extension reaction. It requires no modified primers, thus rendering assay development simple and economical. Despite its limited possibility for multiplex reactions, the throughput of this assay can be high, particularly when a 384-well format is used (Chen et al., 1999).

CONCLUSION

Because alcohol dependence is a common, polygenic disease, candidate gene studies are an important tool to identify the contribution of specific genes to its pathophysiology. Progress in basic science studies of alcohol dependence using various animal models has provided venues to identify novel candidate genes for genetic studies. In combination with gene expression profiling and in vivo gene/antisense transfer techniques, putative genes can be functionally validated. Functionally validated candidate genes can then be tested in clinical genetic association studies. Systematic mutation analysis and genotyping of genetic variations of any candidate gene now has been made possible by recent technological advances, both in bioinformatics and in the laboratory. Therefore, human candidate gene studies assessing carefully selected intermediate phenotypes will contribute to the identification of pathophysiologically causative genes, by identifying every relevant genetic variation, both functional or silent, in a candidate gene and assessing its association with the disease phenotype. This is a fundamentally new situation for candidate gene studies that has only begun to be exploited and that can be expected to lead to exciting results toward the identification of the genetic basis of alcohol dependence.

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