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A Candidate Gene Study of CYP19 (Aromatase) and Male Sexual Orientation

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Aromatase cytochrome P450 (CYP19), which is necessary for the conversion of androgens to estrogens, plays an important role in the sexual differentiation of the brain. To investigate whether differences in the gene encoding the aromatase enzyme influence sexual orientation in men, we conducted linkage, association, and expression analyses in a large sample of homosexual brothers using microsatellite markers in and around CYP19. No linkage was detected, and a gene-specific relative risk of 1.5-fold could be excluded at a lod score of −2. Results of the TDT demonstrated no preferential transmission of any of the CYP19 alleles in this sample. Expression of aromatase mRNA by microarray analysis was not significantly different between heterosexual and homosexual men. These results suggest that variation in the gene for this subunit of the aromatase enzyme complex is not likely to be a major factor in the development of individual differences in male sexual orientation.

KEY WORDS: Homosexuality; sexual orientation; aromatase; CYP19; linkage, expression.

INTRODUCTION

Human male sexual orientation is a complex and variable trait. Although the majority of males are sexually attracted to females, a significant minority (approximately 2%–6%) of males report predominant sexual attraction to males (Diamond, 1993; Laumann *et al.,* 1994; Wellings *et al.,* 1994). Multiple lines of evidence suggest that biological factors play a role in explaining individual differences in sexual orientation (see Mustanski *et al.,* 2002 for review). Neuroanatomical differences have been reported for three brain regions based on sexual orientation in males: the argine vasopressin neuronal population of the suprachiasmatic nucleus (Swaab and Hofman, 1990; Zhou *et al.,* 1995), the third interstitial nucleus of the anterior hypothalamus (Byne *et al.,* 2001; LeVay, 1991), and the anterior commissure (Allen and Gorski, 1992; but see also Lasco *et al.,* 2002). In all instances of significant neuroanatomical differences, gay men were reported to be skewed in the female direction.

Evidence that biological factors may exert their influences prenatally comes from prospective studies demonstrating that the majority of young boys who display feminine behavior develop a homosexual orientation in adulthood, and retrospective studies showing that gay men report more gender nonconforming behavior in childhood (Bailey and Zucker, 1995). The precocity and tenacity of these behaviors suggest an innate predisposition. Prenatal biological influences also are implicated based on evidence for sexual orientation differences in anthropometric traits that

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are believed to be canalized before birth, such as handedness (Lalumière *et al.,* 2000) and finger length (Williams *et al.,* 2000).

Twin studies have consistently shown that male sexual orientation is at least partially heritable (see Mustanski *et al.,* 2002 for review). Two recent population-based studies using systematic ascertainment methods both reported heritability estimates of approximately 60% (Kendler *et al.,* 2000; Kirk *et al.,* 2000). In addition, family studies using a variety of ascertainment strategies have produced a median and mode rate (across studies) of homosexuality in gay brothers of 9%, a figure well above population base rates (see Bailey and Pillard, 1995 for a review). Although no specific genes have been identified, several studies have detected a linkage between male sexual orientation and DNA markers on Xq28 in some but not all families (Hamer *et al.,* 1993, 1999; Hu *et al.,* 1995; Rice *et al.,* 1999). In the only candidate gene study published to date, Macke *et al.* (1993) found no evidence for variations in the androgen receptor gene being related to individual differences in male sexual orientation.

The most influential theory about the ontogeny of homosexuality implicates sex-atypical hormone action during gestation (Ellis and Ames, 1987). This theory is based on animal research that has demonstrated that in many species the developing male brain sexually differentiates under the influence of hormones secreted by the fetal testes (Wilson *et al.,* 1981). The presence of androgen during a species-dependent critical period of development masculinizes the fetal brain and modulates behavioral sexual differentiation (Goy and McEwen, 1980). Behavioral masculinization in a number of species is dependent upon aromatization of androgens to estrogens (Beyer *et al.,* 1976; Hutchison, 1997; Lephart, 1996; Pinckard *et al.,* 2000).

The activity of the aromatase enzyme complex in brain masculinization has been observed in numerous species. It is especially well described, for example, in Japanese quail (Balthazart *et al.,* 1992), zebra finches (Adkins-Regan *et al.,* 1997), rats (Arai, 1972; Luttge and Whalen, 1970), and rams (Pinckard, 2000). The genes for both of the two essential components of the aromatase enzyme complex, aromatase cytochrome P450 and NADPH-cytochrome P450 reductase, are highly conserved among mammals and vertebrates (Conley and Hinshelwood, 2001). Generally speaking, throughout the mammalian class, aromatization of fetal androgens to estrogens plays a critical role in brain masculinization and is necessary for adult male sexual behavior (Pilgrim and Reisert, 1992). In humans, CYP19, the gene encoding the cytochrome P450 component of the aromatase enzyme complex, is expressed in multiple areas of the brain, notably the temporal and frontal neocortex, the hippocampus, and the hypothalamus (Hayes *et al.,* 2000; Stoffel-Wagner *et al.,* 1999). It is believed that sex differences in estrogen levels as a result of aromatization of androgen may explain the sexual dimorphisms found in the hypothalamus (Gorski, 1991). The hypothesis that a feminine differentiation of the hypothalamus is a major cause of homosexuality in men is of long standing (Dorner, 1976; MacLusky and Naftolin, 1981) and has received some empirical support (Byne *et al.,* 2001; LeVay, 1991).

An atypical pathway in the biosynthesis or metabolism of estrogens is a logical element of a model of the development of sexual orientation that incorporates the role of estrogens. The aromatase enzyme is the rate-limiting factor in the conversion of androgens to estrogens; therefore it follows that the enzyme is a potential candidate for an effect during development that is reflected in adult sexual orientation. Recent knockout studies corroborate a role for estrogens in male sexual behavior. Mice deficient in aromatase as a result of a targeted disruption of the CYP19 gene demonstrate severe impairment of sexual behavior consisting of a significant reduction in mount frequency and significant increase in latency to mount (Fisher *et al.,* 1998; Honda *et al.,* 1998) as well as a loss of sexual partner preference (Bakker *et al.,* 2002). Similarly, mice with a knock-out of the estrogen receptor-alpha gene display a reduction in intromissions, increased latency to intromission, and a lack of ejaculation, despite unaffected simple mounting behaviors (Couse and Korach, 1999; Ogawa *et al.,* 1997, 2000).

In human 46,XY individuals with congenital estrogen deficiency the usual male sexual differentiation and pubertal maturation occur, although the mutation results in tall stature and linear growth that continues into adulthood, with delayed bone age and a lack of epiphyseal fusion (Faustini-Fustini *et al.,* 1999). Sterility has been observed as well (Carreau, 2000). Three unique cases of male patients with congenital aromatase deficiency, resulting from mutation of the CYP19 gene, have been published. All reported a heterosexual orientation and male gender identity (Carani *et al.,* 1997; Herrmann *et al.,* 2002; Morishima *et al.,* 1995). However, given the small sample sizes reported in these medical studies, and the clear role for estrogen in masculinization of the brain as evidenced in

animal studies, aromatase still deserves serious consideration as a candidate gene for male sexual orientation.

To test whether sequence variation in the gene for the cytochrome P450 subunit of the aromatase enzyme complex plays a role in individual differences in male sexual orientation, we performed linkage and association analysis on the CYP19 locus in a large sample of gay sibling pairs. Besides variation in the sequence of the aromatase gene, variation in gene expression could affect the development of sexual orientation. Unknown *cis* or *trans* acting factors influencing aromatase gene expression could influence the degree of brain masculinization. To begin addressing this point, we also measured aromatase gene expression levels in cells derived from heterosexual and homosexual men using cDNA microarrays.

METHODS

Participants

The sample for the linkage and association analyses consisted of a total of 439 individuals from 144 unrelated families, of which 135 families had two gay brothers and 9 families had three gay brothers. Fifty-eight of the families included one or more parents and 45 of the families included a heterosexual male or a female sibling who was available for genotyping. The sample included 40 families previously reported by Hamer *et al.* (1993), 33 families previously reported by Hu *et al.* (1995), and 71 previously unreported families.

Subjects were recruited through advertisements in local and national homophile publications. The only criteria for inclusion was the presence of two or more gay male siblings; unlike the previous X chromosome linkage studies (Hamer *et al.,* 1993; Hu *et al.,* 1996), there were no exclusion for nonmaternal transmission or non–sex-limited transmission. The participants were predominantly white, college educated, and of middle to upper socioeconomic status. The mean (SD) age for the gay siblings was 36.98 (8.64). An NCI Clinical Review Subpanel approved the protocols, and each participant signed an informed consent form before interview, questionnaire completion, and donating blood for DNA extraction.

The participants for the expression analysis include nine homosexual subjects from the linkage analysis and eight heterosexual controls who were selected because permanent Epstein-Barr Virus (EBV)–transformed lymphoblastoid cell lines were available. The controls consist of four heterosexual brothers of gay male sibling pairs and four age-matched heterosexual men recruited through an NCI protocol on cigarette smoking (Sabol *et al.,* 1999).

Measures

Sexual orientation was assessed through a structured interview or a questionnaire that included a sexual history and the Kinsey scales of sexual attraction, fantasy, behavior, and self-identification (Kinsey *et al.,* 1948). Each scale ranges from 0 (exclusively heterosexual) to 6 (exclusively homosexual). The mean (SD) of these four scales was 5.65 (0.46) for the gay males. The heterosexual controls used in the expression analysis each had a score of 0 on all four Kinsey scales.

Genotyping

DNA was extracted from peripheral blood by a commercial service (Genetic Design, Greensboro, NC, USA). A polymorphic $(TTTA)_n$ repeat (Polymeropoulos *et al.,* 1991) that begins at the 682 base pair in the human aromatase cytochrome P-450 gene on chromosome 15q21.2 (Chen *et al.,* 1988) was genotyped by radioactive labeling and slab gel electrophoresis. Polymerase Chain Reactions (PCR) were performed on 100 ng of genomic DNA using 12.5 pM of each oligonucleotide primer (Bioserve Biotechnologies, Ltd., Laurel, MD, USA), $dCTP^{32}$, and standard amplification conditions (Weber and May, 1989). PCR products were denatured for 5 min, run on a 6% polyacrylamide gel in an electrophoreses apparatus, dried in a gel vacuum system, and exposed for 20 to 60 min on Kodak film before processing. The most intense band(s) for each allele was used for size determination and was referred to a DNA fragment-sizing ladder for consistent comparison. We report the presence of a total of eight alleles at the CYP19 locus, three more than previously enumerated (Polymeropoulos *et al.,* 1991). Observed frequencies for the eight alleles were as follows: 0.003, 0.048, 0.358, 0.022, 0.007, 0.091, 0.12, and 0.35. Heterozygosity for the polymorphism in our data was 0.72.

In addition to the tetranucleotide repeat polymorphism in CYP19, we include data from an additional four markers from another project involving the same subjects. A semiautomated analysis of these flanking microsatellite genotypes was conducted in the Laboratory of Biochemistry Section on Gene Structure and Regulation of the National Cancer Institute, Bethesda, Maryland. An 11.25-μl total reaction volume consisted of 0.25 mM dNTP (Pharmacia Biotech, Little Chalfont,

Bucks, UK), 1 × buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$), 0.675 units *AmpliTaq Gold* DNA Polymerase (PE Biosystems, Foster City, CA, USA), 1.0 mM $MgCl_2$, and ~60 ng DNA. Primer concentrations were determined empirically and varied according to their membership in particular ABI PRISM Linkage Mapping Set panels. The PCR conditions consisted of an initial denaturation at 95°C for 12 min; 10 cycles of denaturation, annealing, and extension at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, respectively, followed by 20 cycles of denaturation, annealing, and extension at 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s, respectively; followed by a 10-min extension at 72°C.

PCR products were sized with the GeneScan version 3.1.2 program (PE Biosystems, Foster City, CA, USA), and genotypes were studied and assigned with the Genotyper version 3.6 program (PE Biosystems). A PCR product from a DNA reference sample (Centre d'Etudes du Polymorphisme Humain [CEPH] 1347-02) was included in each run to monitor sizing conformity.

Expression Analysis

White blood cells from peripheral blood of the participants was infected with EBV to create lymphoblastoid cell lines (Genetic Design). Total RNA was extracted from the cultured cells using TriZOL (Gibco/BRL/Life Technologies, Carlsbad, CA, USA) and 50 μg was reverse transcribed using SuperScript II (Gibco/BRL) in the presence of Cy3- or Cy5-labeled dUTP as described (Eisen and Brown, 1999). Each sample was hybridized on a single array against a reference pool consisting of the RNA of 33 male and female subjects labeled with the other dye. Microarray slides were obtained from the Advanced Technology Center, National Cancer Institute, and contained 9984 clones from the UniGEM2 collection (Incyte Genomics, Palo Alto, CA, USA). The microarrays were prehybridized for 1 h at 42°C in 20μ1 of 5 × SSC, 0.1% SDS, and 1% BSA, then washed in deionized water followed by 100% isopropanol. Slides were hybridized to a mixture of the Cy3- and Cy5-labeled cDNA in 22μ1 of 25% formamide, 5 × SSC, and 0.1% SDS for 12 to 16 h at 42°C, washed (Eisen and Brown, 1999), then scanned on a GenePix 4000A scanner (Axon Instruments, Foster City, CA, USA). The resulting images were analyzed using GenePix Pro v3.0 software (Axon Instruments) and the Cy3 and Cy5 signal intensities were normalized for each sample using the BRB ArrayTools developed by Dr. Richard Simon and Amy Peng. Data filters based on signal intensity and spot quality were used to exclude less reliable spots. The normalized Cy5/Cy3 intensity ratio for the array spot containing CYP19 cDNA was used in the analysis.

RESULTS

Linkage Analysis

We initially performed single-point linkage analysis using a marker located within the CYP19 coding sequences. Sexual orientation was treated as a dichotomous trait, and all independent sib pairs within each family were included. This analysis gave negative LOD scores for models both assuming and not assuming dominance.

To increase the power of the linkage test, we performed multipoint analysis using data on four additional markers that flank CYP19 on chromosome 15q; these loci were genotyped as part of a concurrent genome screen project. The statistical analysis was performed with MAPMAKER/SIBS (Kruglyak and Lander, 1995) using the Haldane mapping function (Haldane, 1919).

Information content refers to the extent that the available data extracts the full identity-by-descent (IBD) allele sharing status at a given map position. At CYP19, the locus of interest here, the extraction was 82%. This relatively high information content, despite the absence of parental data in many of the pedigrees, is due to the high average heterozygosity of the markers (0.77) and the use of multipoint information.

Figure 1 shows the linkage mapping of the region under the assumption of no dominance variance. The value of λ_s, the gene-specific relative risk ratio for a sibling compared to a member of the general population (Risch, 1990), was allowed to vary from 1.5- to 5-fold. The LOD scores were consistently negative throughout the mapped interval, falling from −2 at $\lambda_s = 1.5$ to −14.2 at $\lambda_s = 5$.

The multipoint analysis of the region produced estimated allele sharing proportions at the CYP19 locus of 0.25, 0.50, and 0.25 for 0, 1, and 2 alleles, respectively. These results are no different than the expected Mendelian sharing of 25%, 50%, and 25% if this gene was unrelated to male sexual orientation.

Association Analysis

A transmission disequilibrium test was performed using the sib_tdt algorithm in ASPEX v. 2.4 (Hinds and Risch, 1996). Table I shows the results for each of the eight alleles split by paternal and maternal transmission,

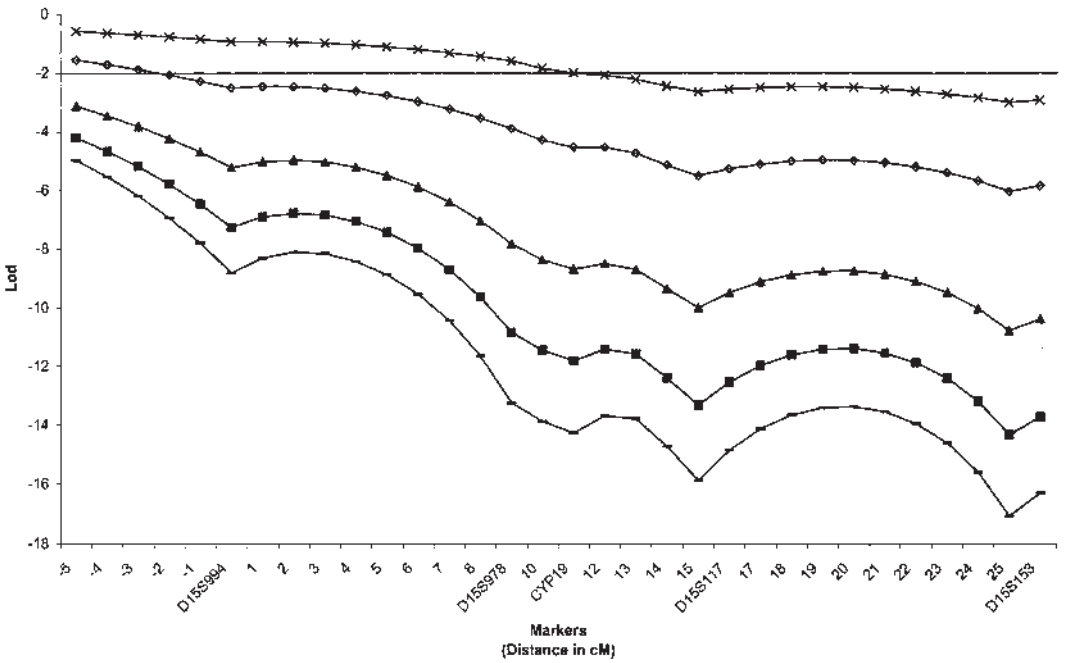

Fig. 1. Multipoint gay sib pair linkage analysis including the polymorphic repeat in the CYP19 gene and the four flanking markers (indicated with a D prefix). The following relative risk values were included in the analyses: (**x**) = 1.5; (◇) = 2.0; (▲) = 3.0; ■ = 4.0; (—) = 5.0.

as well as the combined evidence. All individual allele results were nonsignificant (lowest $p > 0.55$), as were the results for the overall TDT ($p = 0.21$).

Expression Analysis

To determine the expression levels of the CYP19 gene in homosexual compared to heterosexual males, RNA was prepared from lymphoblastoid cell lines and hybridized to a microarray. Figure 2 shows a scatter plot of the expression ratios (plotted as $\log_2$ values) for 9 gay men and 8 heterosexual controls compared to a reference sample prepared from the mixed RNA of 33 male and female subjects. The relative expression levels were 0.982 (±0.178) for the homosexual subjects and 0.822 (±0.143) for the heterosexual subjects. The ratio of expression values was 1.19, with a 95% confidence interval of 1.00 to 1.43. There was no significant association between the length of the CYP19 tetranucleotide repeat and expression level.

Table I. Results of TDT Analysis

			Paternal			Maternal			Combined		
Allele	N	%	TR	NT	χ^2	TR	NT	χ^2	TR	NT	χ^2
1	0	0	0	0	0.00	0	0	0.00	0	0	0.00
2	5	2.8	1	1	0.00	3	5	0.50	4	6	0.40
3	62	35.2	10	10	0.00	21	21	0.00	38	38	0.00
4	4	2.3	1	3	1.00	3	1	1.00	4	4	0.00
5	2	1.1	0	2	2.00	0	2	2.00	0	4	4.00
6	19	10.8	5	7	0.33	8	13	1.19	13	20	1.48
7	21	11.9	5	8	0.69	9	12	0.43	15	21	1.00
8	63	35.8	23	14	2.19	24	14	2.63	55	36	3.97
Total			$\chi^2 = 6.21,\ p = 0.55$			$\chi^2 = 7.75,\ p = 0.46$			$\chi^2 = 10.85,\ p = 0.21$		

Note: All individual allele results nonsignificant (lowest $p = 0.56$). % heterozygosity = 80.7, % typed = 65%.
N = Number of times allele is seen in parents, and % is the % frequency. TR = Number of times the allele was transmitted; NR = number of times the allele was not transmitted.

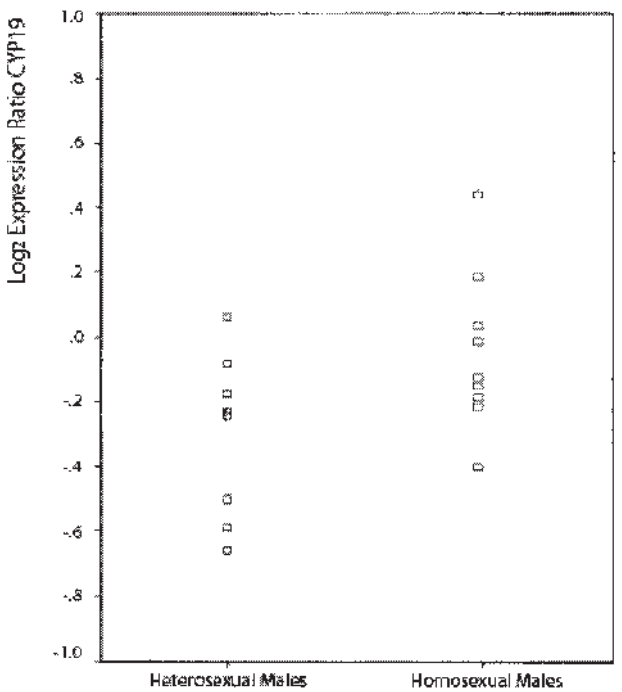

Fig. 2. Expression analysis of CYP19. The $\log_2$-value of the expression ratio for CYP19 in EBV-transformed lymphoblast cells for eight heterosexual and nine homosexual males. A $\log_2$-value of 1 corresponds with a 2-fold increase in expression, a $\log_2$-value of -1 corresponds with a 2-fold decrease of expression.

DISCUSSION

CYP19 is a strong candidate for a role in sexual orientation. It plays a key role in a pathway known to be important for sexual differentiation of the brain, and animal knock-out models display clear alterations in sexual behavior and partner preference. Nevertheless, the linkage, association, and expression data reported here suggest that this gene is unlikely to play a significant role in the generation of individual differences in human male sexual orientation.

Multipoint linkage analysis showed no relationship between the inheritance of the CYP19 locus and sexual orientation in a large sample of gay sib pairs. The usual exclusion criterion of lod $= -2$ was reached at a gene-specific relative risk factor of $\lambda_s = 1.5$, which is well below the estimated overall relative risk factor of 3.0 to 4.0 (Bailey *et al.,* 1999). The lack of a relationship was mirrored by the results of the TDT, which found no association for any of the alleles. These results rule out the possibility that CYP19 is the predominant locus contributing to variation in male sexual orientation within this sample, although a minor role cannot be excluded.

As with any analysis reporting a null result, it is important to determine if the sample provided sufficient power to detect the hypothesized effect. Based on the power calculations reported by Risch (1990), which utilized a lod score of 3 as the criterion for linkage significance, it is possible to estimate the power to detect an effect of the aromatase gene on variation in male sexual orientation with our sample. Given that the primary marker was located within the CYP19 gene, the recombination fraction was set to 0 for the purpose of power calculations. With n pairs $= 153$ (counting triplets as 2 pairs) and a highly informative marker (based on information content), the power is estimated to be approximately 95% for an overall relative risk of 3. Based on these calculations, this sample should provide sufficient power for detection of a major role for the aromatase gene in determining sexual orientation given the above assumptions.

Although the linkage and association analyses indicated that inherited variations in the aromatase gene sequence do not play a role in individual differences in sexual orientation within this sample, it was still theoretically possible that unknown *trans*-acting factors influenced brain masculinization by altering aromatase gene expression. To explore this possibility, we performed microarray analysis of CYP19 mRNA levels in cells from homosexual compared to heterosexual males. Although there was a trend for more CYP19 mRNA in the gay male samples, the difference between the two groups was less than 20%, which was not statistically significant and seems unlikely to be biologically meaningful.

There were several important limitations of the present study. First, we did not include any analysis of the nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase subunit of the aromatase enzyme complex. This protein, which is encoded by a gene on 7q11.23 (Shephard *et al.,* 1989), is an ubiquitous electron donor for oxygenase enzymes performing a variety of functions throughout the body. Therefore it seemed unlikely that organisms could tolerate genetically significant differences in the coding sequences or expression of this gene.

A second limitation, common to all human genetic studies, is that we were limited by the DNA sequence variability present in the particular population we studied. Thus our results do not show that aromatase per se plays no role in the development of sexual orientation, but rather that naturally occurring variations in the CYP19 gene do not play a major role in individual differences in this trait.

Third, our expression studies were limited by the use of cultured lymphoblasts from a limited number of adult subjects. Thus, although the data show that gay men do not frequently contain *cis* or *trans*-acting factors that universally alter CYP19 expression in all cell types, they do not address the possibility of more subtle changes in particular cell types or developmental-specific effects. For obvious logistic and ethical reasons, it is not feasible to analyze embryonic brain cells from human subjects; this question would be more appropriately addressed in an animal model.

SUMMARY

These studies do not support a major role of CYP19 in human male sexual orientation. Nevertheless, given the consistent evidence from animal studies (Wilson *et al.,* 1981) and human clinical studies (see Mustanski *et al.,* 2002 for a review), suggesting a role for sex hormones in the development of sexual orientation, future candidate gene studies should continue to consider loci that play a role in this axis.

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