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Linkage Disequilibrium at the Angelman Syndrome Gene *UBE3A* in Autism Families

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Autistic disorder is a neurodevelopmental disorder with a complex genetic etiology. Observations of maternal duplications affecting chromosome 15q11–q13 in patients with autism and evidence for linkage and linkage disequilibrium to markers in this region in chromosomally normal autism families indicate the existence of a susceptibility locus. We have screened the families of the Collaborative Linkage Study of Autism for several markers spanning a candidate region covering ~ 2 Mb and including the Angelman syndrome gene (*UBE3A*) and a cluster of γ-aminobutyric acid ($GABA_A$) receptor subunit genes (*GABRB3*, *GABRA5*, and *GABRG3*). We found significant evidence for linkage disequilibrium at marker *D15S122*, located at the 5′ end of *UBE3A*. This is the first report, to our knowledge, of linkage disequilibrium at *UBE3A* in autism families. Characterization of null alleles detected at *D15S822* in the course of genetic studies of this region showed a small (~ 5-kb) genomic deletion, which was present at somewhat higher frequencies in autism families than in controls.

Key Words: autism, 15q11–q13, UBE3A, linkage disequilibrium, deletion

INTRODUCTION

Classic autism, or autistic disorder (AD; MIM 209850), is one of five pervasive developmental disorders sharing several core features that define a broader autism phenotype [1]. Core characteristics include deficits in language development and communication, paucity of reciprocal social interaction, and patterns of repetitive or restricted behaviors and interests. AD is distinguished from other autism-spectrum disorders such as Rett or Asperger syndromes by the number, age of onset, and progression of core features, or by the presence of additional elements. Autism has a population prevalence of ~ 1 in 2000 with a male:female ratio of 4:1 [2]. Impairments are usually detected in early childhood and persist through adulthood. Neurological and neuropsychiatric findings often seen in the autistic population may include mental retardation, hypotonia, poor motor coordination in young children, motor stereotypies, obsessive–compulsive and self-injurious behavior, and abnormal electroencephalographic patterns or epilepsy.

Although autism is seen as a component of several neurodevelopmental, single-gene disorders such as tuberous sclerosis, Angelman, Cornelia de Lange, and fragile X syndromes [3–5], idiopathic autism is thought to be an oligogenic, multifactorial disorder [6]. Evidence from twin and family studies supports the idea of a strong genetic etiology; reports estimate heritability as high as 90% [7] and sibling recurrence risks 50–100 times the population prevalence [8]. Although these studies indicate that AD is one of the most strongly genetic neuropsychiatric disorders known, they also emphasize its genetic complexity and heterogeneity.

Involvement of chromosome 15q11–q13 has been indicated in AD etiology based on two lines of investigation: observations of chromosomal duplications in a small percent of autistic individuals and evidence of linkage and linkage disequilibrium (LD) in some AD family data sets. Defects in this chromosomal

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region are associated with two other neurodevelopmental disorders that share certain features with autism. Specifically, common interstitial deletions of 15q11–q13 result in Prader-Willi syndrome (PWS; MIM 176270) or Angelman syndrome (AS; MIM 105830), depending on the parental origin of the affected chromosome [9]. Thus, opposite patterns of genomic imprinting underlie PWS and AS. Candidate genes for PWS are expressed in a paternally specific way [10] and the AS gene *UBE3A*, encoding the E6-AP ubiquitin–protein ligase, is expressed in a maternally biased way in the brain [11–13]. Duplications affecting 15q11–q13 are the most common chromosomal abnormalities seen in autistic populations. Autism-related duplication events involving proximal 15q occur as either interstitial duplications of 15q11–q13, reciprocal to PWS/AS deletions, or supernumerary pseudodicentric inverted duplicated marker chromosomes 15 [14,15]. The latter are commonly referred to as inv dup(15) marker chromosomes. Interstitial duplications result in a total of three copies of the 15q11–q13 interval, whereas inv dup(15) marker cases result in a total of four copies of a larger interval. Association of duplications with autism-spectrum phenotypes depends on parental origin of the duplication [16–18]. Paternal duplications are associated with comparatively benign nonautistic phenotypes, whereas duplications of maternal origin result in or confer substantial risk for development of autistic phenotypes. Increased gene copy strongly indicates a gene dosage effect, with maternal specificity indicating involvement of *UBE3A* or other maternally expressed genes such as *ATP10C*, recently shown to map to this region [19–21]. Other functional candidate genes in this region include a cluster of γ-aminobutyric acid ($GABA_A$) receptor subunit genes (*GABRB3*, *GABRA5*, and *GABRG3*).

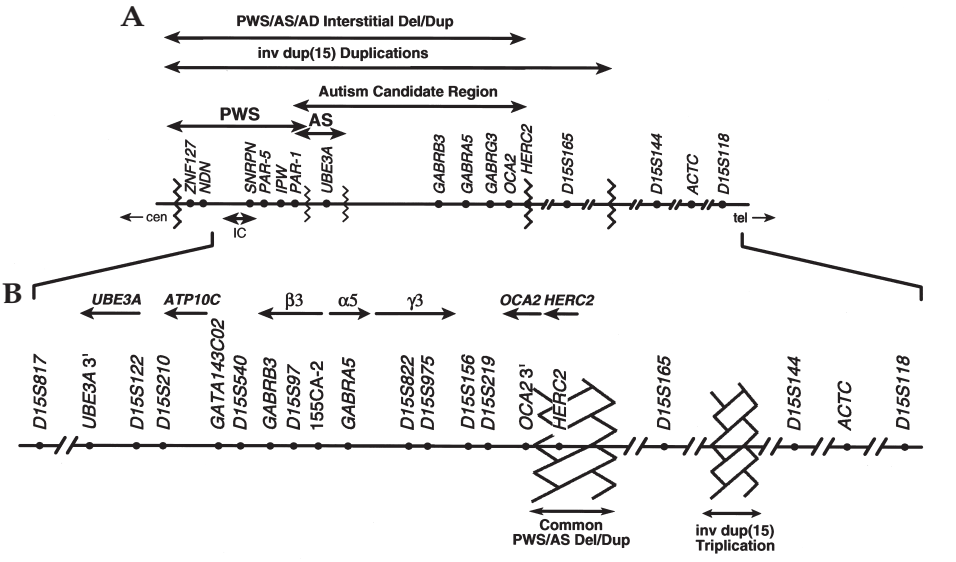

FIG. 1. The 15q11–q13 autism candidate region. (A) Low-resolution view of the 15q11–q13 region. Horizontal arrows indicate intervals affected by PWS and AS interstitial deletions and AD interstitial duplications, the 15q11–q13 region duplicated in inv dup(15) marker chromosomes, and PWS, AS, and AD candidate regions. IC, 15q imprinting center. Gene loci are above the linear map, and hatched jagged lines indicate positions of chromosomal breakpoints associated with deletions and duplications, related to intervals identified above the map. Parallel slanted lines (/ /) designate interruptions of the normal scale of the map and may indicate substantial intervening sequence. (B) Higher-resolution view of the AD candidate region spanning from *UBE3A* to the common PWS/AS/AD deletion/duplication breakpoint and extending, with interruptions through the inv dup(15) breakpoints and linked markers in autism data sets. Gene loci and microsatellite markers are above the linear map, and horizontal arrows indicate transcriptional orientation.

Genetic evidence for the existence of a susceptibility locus in this region is mixed. One group detected suggestive evidence for linkage in this region, with maximum two-point LOD (1.37) and nonparametric Z values (1.78; $P = 0.03$) at a marker D15S217 within the narrow (interstitial) duplication interval [22]. The Collaborative Linkage Study of Autism (CLSA) found only relatively weak evidence for linkage at marker *D15S975* (maximum multipoint heterogeneity lod (MMLS/het) score = 0.51), within the duplication interval, and at marker *ACTC* (MMLS/het = 0.54), located ~ 20 cM telomeric [23]. The Paris Autism Research International Sibpair Study detected a maximum multipoint lod score (MLS) of 1.1 at *D15S118* near *ACTC* but telomeric to the minimal duplication interval [24]. Two other collaborative studies, the International Molecular Genetic Study of Autism consortium and one from Stanford, did not detect linkage to this region in their respective data sets [25,26]. In general, linkage studies in autism have identified few common regions of linkage across data sets, with a locus at 7q31 being a notable example of a susceptibility locus detected by most groups. This pattern arguably reflects the genetic heterogeneity inherent in autism, and the possibility of relative weak genetic effects, compounded by hypothetical epistasis and/or gene/environment interactions.

Several groups have detected significant LD at markers in the 15q11–q13 duplication interval. Cook and colleagues reported LD at a marker (155CA-2) within the $GABA_A$ β3 receptor subunit gene (*GABRB3*) [27]. The Duke University group was unable to replicate the finding at *GABRB3* 155CA-2 but detected disequilibrium at the nearby marker GABRB3 located ~ 60 kb 3′ and centromeric to the gene [28]. The International Molecular Genetic Study of Autism consortium and Stanford groups failed to detect evidence of LD in their respective data sets [29,30].

Whether a common locus might be involved in the etiology of 15q11–q13 duplication-associated autism and inherited

TABLE 1: T_{SP} analysis for 15q11–q13 markers in autism

Marker	*df*[a]	Overall χ^2	*P* value	No. of parents[b]
D15S122	10	27.565	0.0038	9, 121
D15S210	12	6.516	0.8879	10, 102
GATA143C02	4	6.582	0.1597	5, 96
D15S540	3	5.800	0.1218	4, 47
GABRB3	12	11.427	0.4927	8, 124
D15S97	12	14.500	0.2699	9, 135
GABRB3-155CA2	13	9.798	0.7104	11, 124
D15S511	10	12.584	0.3214	9, 130
GABRB3-10C/G	1	0.552	0.4576	0, 60
GABRA5	8	5.655	0.5805	12, 120
D15S822	16	18.343	0.3675	6, 142
D15S975	6	7.042	0.4246	6, 105
D15S156	3	17.263	0.0448	5, 92

[a]Degrees of freedom.
[b]Number of heterozygous parents in triads and sib-pair families, respectively, used for each test.

susceptibility in chromosomally normal individuals is unknown. Here we sought to address this question by testing for evidence of linkage disequilibrium in the most narrow candidate region based on chromosomal duplications. A prioritized candidate region based on maternal-specific chromosomal duplication excludes the PWS region and therefore the proximal third of the 15q11–q13 duplication/deletion interval (Fig. 1). Maternal specificity indicates involvement of *UBE3A*, *ATP10C*, or other, perhaps unknown, maternally expressed genes in the distal two-thirds of the 15q11–q13 interval. Genetic evidence seems to indicate the distal one-third of this interval, which contains the $GABA_A$ receptor subunit cluster. To investigate the 15q11–q13 region more fully, we followed up the initial CLSA genomic screen by analyzing many additional markers in a larger collection of families for evidence of linkage disequilibrium. We carried out genetic studies using markers spanning the most narrow duplication-associated candidate region, spanning from the AS gene *UBE3A* through the cluster of $GABA_A$ receptor subunit genes. Additionally, we report here the molecular characterization and analysis of a genomic deletion associated with null alleles detected at *D15S822* in the region of interest.

RESULTS

To carefully analyze the CLSA data set for evidence of linkage disequilibrium in this region, we chose several additional markers beyond those used in the initial genomic linkage screen. We selected a total of 13 markers from the candidate region, spanning ~ 2 Mb from *UBE3A* through the $GABA_A$ subunit cluster. We typed markers in DNA samples from the 75 sib-pair families reported in the initial screen and in samples from 19 sib-pair families collected subsequently. The results from linkage disequilibrium analysis using the T_{SP} statistic (see Materials and Methods) for the complete data are shown in Table 1, which shows nominal *P* values; results > 0.05 were considered non significant. We found significant linkage disequilibrium at *D15S122* in the overall analysis (χ^2 = 27.565; 11 *df*; *P* = 0.0038). *D15S122* is located at the 5′ end of *UBE3A*, within an intron in the 5′ untranslated region. Given the relevance of genomic imprinting to this region, we stratified the data set for maternal and paternal transmissions using the T_{SP} test (Table 2). Results of this stratified analysis for *D15S122* approach nominal significance for maternal transmissions (χ^2 = 18.028; 10 *df*; *P* = 0.0545). To further characterize the association at *D15S122*, transmissions are shown for each of the alleles at this locus (Table 3). These results indicate that the 155-bp allele is preferentially transmitted to affected siblings, whereas the 147-bp allele is preferentially not transmitted in negative association. We found a significant maternal effect for the preferential transmission of the 155-bp allele, whereas maternal and paternal non-transmissions of the 147-bp allele did not independently achieve significance (data not shown).

We found nominally significant results at *D15S156* (χ^2 = 17.263; 3 *df*; *P* = 0.0448) in the overall analysis (Table 1) and at *D15S540* when the data were stratified on paternal transmissions (χ^2 = 10.176; 3 *df*; *P* = 0.0171; Table 2). However, neither observation remained significant after Bonferroni correction for multiple tests. *D15S540* is ~ 200 kb centromeric to the 3′ end of *GABRB3* and *D15S156* is approximately the same distance telomeric to the 3′ end of *GABRG3* (Fig. 1).

We calculated MMLS/het scores for the CLSA chromosome 15 candidate region defined by the markers *D15S817* through *D15S118* (Fig. 1). Compared with the initial CLSA genomic screen (75 families), we found slightly increased, although still modest, lod scores with a peak MMLS/het value of 0.75 at *D15S975* (data not shown).

Given previous observations of increased recombination in this region in autism families [22], we analyzed sex-averaged and sex-specific recombination in the CLSA autism families, compared

TABLE 2: Parental-specific T_{SP} analysis for 15q11–q13 markers in autism

Marker	*df*[a]	Father χ^2	*P* value	No. of parents[b]	*df*[a]	Mother χ^2	*P* value	No. of parents[b]
D15S122	7	10.707	0.1519	3, 56	10	18.028	0.0545	6, 53
D15S540	3	10.176	0.0171	1, 19	2	0.816	0.6649	3, 22
D15S156	7	12.458	0.0865	2, 40	5	7.559	0.1823	1, 26

[a]Degrees of freedom.
[b]Number of heterozygous parents in triads and sib-pair families, respectively, used for each test.

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TABLE 3: Allele-specific transmissions and non-transmissions for *D15S122*, with T_{SP} statistic and χ^2 *P* values for each test

	Triads		Pairs				
Allele	T[a]	NT[b]	T[a]	NT[b]	H*[c]	T_{SP}	*P* value
137	0	0	2	0	1	1.00	0.317
143	2	0	5	9	1	0.22	0.637
145	4	1	46	40	10	0.78	0.377
147	0	4	60	76	9	5.17	0.023
149	1	1	69	55	16	0.94	0.331
151	0	1	18	38	1	2.27	0.132
153	1	1	10	6	3	0.89	0.346
155	1	1	27	11	10	5.90	0.015
157	0	0	1	3	0	1.00	0.317
159	0	0	3	1	1	1.00	0.317
161	0	0	0	2	0	1.00	0.317
163	0	0	1	1	0	0.00	1.000

[a]Number of times a parent, heterozygous for the allele, transmitted the allele to an affected child.
[b]Number of times a parent, heterozygous for the allele, did not transmit the allele to an affected child.
[c]Number of times a parent, heterozygous for the allele, transmitted the allele to both affected siblings in a sib-pair family.

with Centre d'Etude du Polymorphisme Humain (CEPH) families [22] using CRIMAP. Analysis for the region from *D15S817* to *D15S219* (Fig. 1) indicated somewhat reduced recombination in the autism families compared with that of the CEPH families in the sex-averaged map (15.1 cM versus 16.8 cM; χ^2 = 18.25; 7 *df*; *P* = 0.0009; Fig. 2). The sex-specific maps (Fig. 2) showed a significant difference, with reciprocal patterns of reduced female (10.9 cM versus 16.2 cM) and increased male (19.1 cM versus 16.9 cM) recombination in autism versus CEPH families (χ^2 = 27.85; 14 *df*; *P* = 0.0148). The overall interval covers a physical distance of ~ 2.5 Mb, indicating much higher overall recombination compared with genome-wide estimates of 1 cM per 1 Mb. The significant differences derive from the interval between *D15S817* and *D15S122*, which includes the *UBE3A* transcriptional unit.

In the course of the CLSA genomic screen and studies reported here, we found several examples of informative null alleles at the tetranucleotide microsatellite marker *D15S822*; this marker is located within the *GABRG3* transcriptional unit (Fig. 1). Null alleles were indicated based on the observation of families in which one or both siblings failed to inherit an informative allele from one parent with a homozygous genotype (data not shown). Such a result theoretically can be caused by a failure of PCR amplification because of polymorphism at the primer binding site(s) or by genomic deletion encompassing the marker. As this phenomenon was not found in other disease-screening projects, it may represent a deletion specific to this population, which could have a functional effect or be

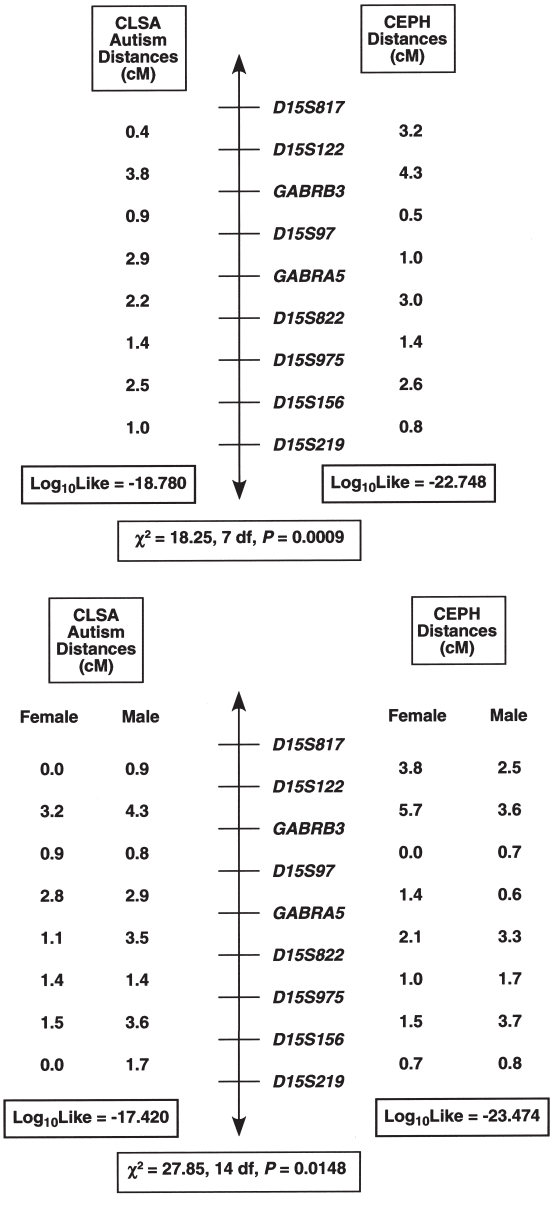

FIG. 2. Comparison of 15q11–q13 genetic maps from CLSA autism and CEPH families. Distances in cM between markers in CLSA autism and CEPH data sets were estimated using CRIMAP. $\chi^2 = 4.6[\text{Log}_{10}\text{Likelihood}_{AD} - \text{Log}_{10}\text{Likelihood}_{CEPH}]$, and degrees of freedom (*df*) are based on the number of distances being compared. Likelihoods were calculated on the CLSA data set using CRIMAP; first the distances from the AD data were estimated, then the map was fixed at the CEPH distances to obtain a CEPH likelihood for the comparisons.

in linkage disequilibrium with a susceptibility allele. PCR amplification using a new set of primers flanking the original amplimer (GenBank acc. no. G07911) and subsequent observation of identical results permitted exclusion of the possibility of primer-site polymorphism (data not shown). To test for deletion, we cloned a 4.8-kb *Pst*I genomic fragment containing

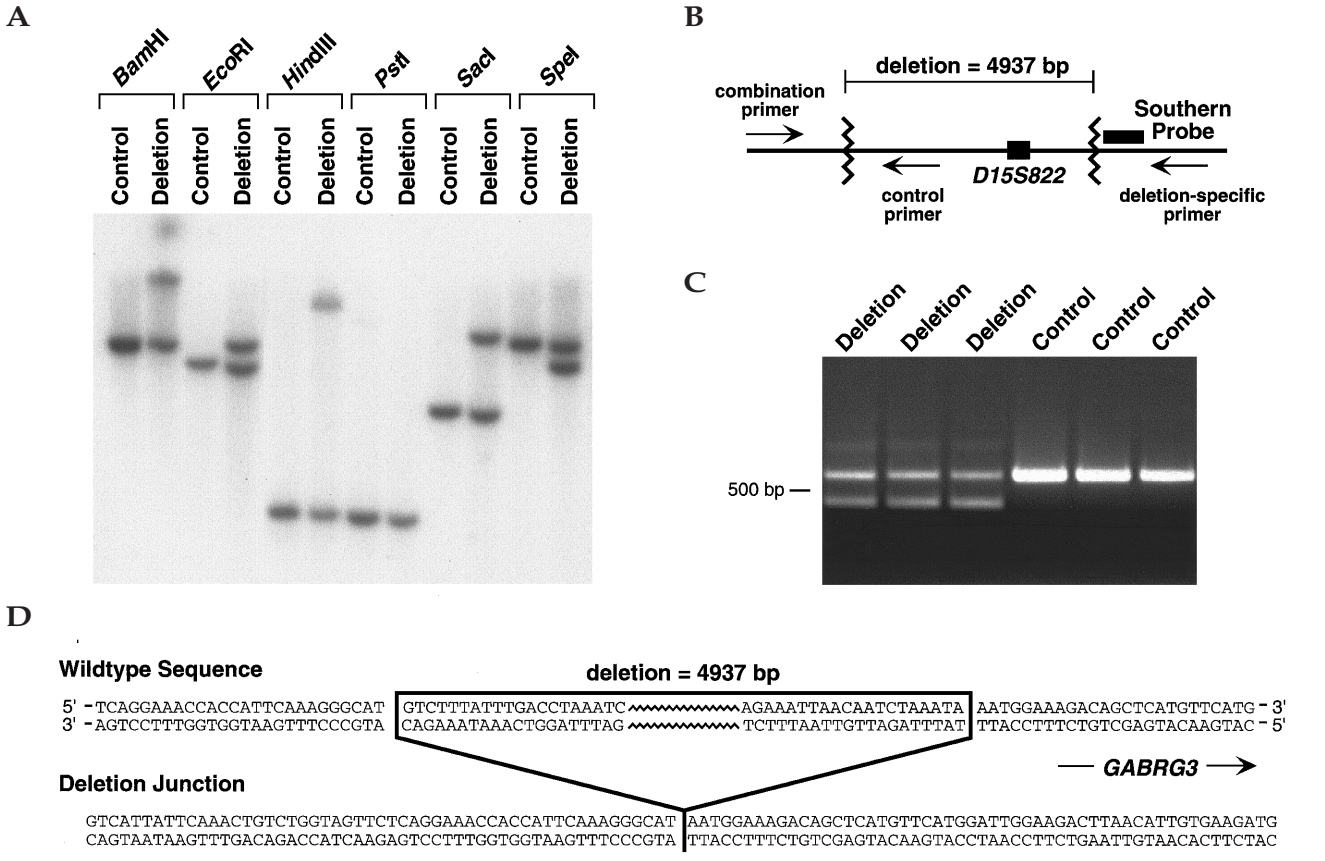

FIG. 3. Molecular analysis of genomic deletion at *D15S822*. (A) Southern blot detection of deletion junction fragments at *D15S822*. Genomic DNA samples are from a control and an individual with an informative null allele at *D15S822*. Above blot, restriction enzymes. For the deletion sample digested with *Pst*I, a lower-molecular-weight variant fragment has been cropped from this image. (B) Deletion region, showing positions of the *D15S822* microsatellite marker, PCR primers for amplification of deletion and non-deletion chromosomes, and probe for Southern blot analysis. Jagged lines indicate position of deletion breakpoints. (C) Duplex PCR screening assay. PCR product sizes are 604 bp for a non-deletion chromosome and 429 bp for a deletion chromosome. (D) Sequence of deletion junction, showing sequence from non-deletion and deletion chromosomes. The deletion removes 4937 bp of sequence, including the *D15S822* marker locus.

D15S822 from P1-derived artificial chromosome (PAC) clone 69I9 [31]. We obtained genomic DNA from families or individuals with apparent null alleles and controls, digested it with *Pst*I, and hybridized it to the 4.8-kb PAC fragment in a Southern blot analysis using conditions of repeat suppression. All samples showed the expected 4.8-kb fragment, but samples with null alleles demonstrated an additional common variant fragment (data not shown). We suspected this represented a deletion junction fragment, but to rule out the possibility of a restriction-site polymorphism, we tested several enzyme digests in combination with smaller probes from either end of the subcloned fragment (Fig. 3A). We detected variant fragments with all enzymes, indicating that a genomic deletion was the most likely explanation (Fig. 3B).

Comparative restriction mapping of deleted and non-deleted alleles using sequence of an overlapping PAC (476I9; GenBank acc. no. AC006602) containing *D15S822* and Southern blot data indicated a deletion of ~ 5 kb. Based on the PAC sequence and the estimated position of the deletion, we designed PCR primers to amplify a product specific for the presumptive deletion allele. PCR amplification demonstrated a 2.5-kb PCR product in null allele samples previously showing variant Southern blot fragments, but not in other samples, confirming the existence of a deletion (data not shown). We sequenced PCR products and identified the precise location of the deletion junction (Fig. 3D); the junction brings together nucleotide positions 58,632 and 63,570 in the 476I9 sequence. Analysis of this sequence showed the deletion occurs within an interrupted long interspersed nuclear element (LINE) repetitive sequence. To facilitate the analysis of this deletion in a larger collection of samples, we developed a duplex PCR assay (Fig. 3B) using three primers, which amplified both nondeleted and deleted alleles in a single reaction (Fig. 3C). We applied the duplex assay to the CLSA samples and samples from a group of autism families from the Duke and Greenwood Genetic Center groups. We found the deletion in 15 of 1295 (1.2%) unrelated chromosomes in this large autism data set, but only in 2 of 606 (0.3%) unrelated

chromosomes in a control Caucasian sample ($P = 0.058$). This analysis demonstrated a small number of previously unrecognized (uninformative) null alleles. Segregation analysis showed that the deletion did not always segregate with phenotype in simplex or multiplex families. In eight of ten examples of paternal carriers, the deletion was transmitted, compared with two of five instances of transmission for maternal carriers. This slightly higher value for paternal transmission was not significantly more frequent than would be expected based on the null hypothesis of no linkage ($P = 0.167$). Analysis of all possible transmissions to affected and unaffected family members using the pedigree disequilibrium test as a test of association demonstrated no significant effects.

The *D15S822* marker and corresponding deletion is within the *GABRG3* transcriptional unit, located within a large (~ 200-kb) intron. Analysis of sequence from PAC 476I9 showed several expressed sequence tags corresponding to a transcript (UniGene Hs.112071) located about 8 kb from the deletion. All expressed sequence tag clones were derived from testis cDNA libraries, and northern blot analysis showed a moderately abundant transcript of ~ 1 kb exclusively in testis (data not shown). Detailed analysis of the sequence of this transcript indicated it is noncoding and database searching showed no obvious function.

Discussion

We found linkage disequilibrium between the marker locus *D15S122* and autism in the CLSA families. *D15S122* is located at the 5′ end of the AS gene *UBE3A*, ~ 4 kb transcriptionally downstream (centromeric) from 5′ untranslated exons OP2a and OP2b and the corresponding CpG island [13]. Given that *UBE3A* is subject to imprinted, maternal-specific expression in brain, and that association of maternal-specific 15q11–q13 duplications with autism-spectrum phenotypes has been described, we examined maternal and paternal transmissions separately for this and other markers in the region. Although the data show a trend toward a maternal effect based on nominal results ($P = 0.0545$), these data should be interpreted cautiously. When corrected for testing multiple markers and for examining male/female transmissions, the result was not significant.

These results are the first report to our knowledge of LD at *UBE3A* in autism families. A previous report of LD mapping by Cook, *et al*., included *D15S122* and other markers at *UBE3A* (*D15S10*, OP2, 3A, and *UBE3A* e1a); however, no significant association was detected in their data set [27]. A substantial contrast between our study and that of Cook, *et al*., concerns the structure of families studied. Our study consisted of 94 sib-pair families, whereas the Cook, *et al*., report included 6 sib-pairs, 13 parent–child pairs, and 125 trios. Given the possibility for the involvement of or bias toward distinct genetic mechanisms in contributing to overall susceptibility in trios compared with that in sib-pairs, contrary results would not necessarily be unexpected. A study by Martin and colleagues analyzed linkage disequilibrium specifically in *GABRB3* and *GABRA5* and did not include *UBE3A* [28]; therefore, a direct comparison is not possible. Another difference between our study and that of Cook, *et al*., involves the method of analysis: Cook, *et al*., used the multiallelic transmission disequilibrium test. The T_{SP} differs from this test in that information from both affected sibs in a sib-pair is used instead of selecting only one affected individual from each family.

Although the association at *D15S122* was significant, it was only slightly below the significance threshold after Bonferroni correction ($P = 0.0494$). It is possible that involvement of multiple genes, each with a modest genetic effect, may underlie autism etiology [26]; data from twin studies and linkage results from various groups are consistent with this hypothesis. The modest level of evidence from this region might be a chance finding, although it is also consistent with a gene of moderate effect in a small number of families, or a gene in epistasis with other genes, such as that proposed for a locus at 7q31. "Parsing" of highly significant effects may require phenotypic subset analysis of the genetic data and analysis for potential gene–gene interactions. Subset analysis of the CLSA families for language phenotypes has recently produced evidence of increased lod scores on chromosome 7 [32]; however, similar analyses have not yet been made for chromosome 15. A recent report describing analysis for epigenetic and epistatic effects with chromosome 7 and 15 loci supports the idea that analyzing for such effects may increase the power to detect genetic susceptibility in autism [33], and similar studies of the CLSA data set are underway to address these issues.

Our observations of increased male and decreased female recombination in 15q11–q13 contrast with the report from Bass and colleagues [22]. It is unclear why these differences exist, because sample makeup and sizes are similar. Further studies may shed light on this issue.

UBE3A is a logical positional and functional candidate gene for involvement in autism susceptibility in the 15q11–q13 region. Specificity of maternal versus paternal duplications of 15q11–q13 in association with autistic phenotypes indicates an imprinting effect related to gene dosage. *UBE3A* and *ATP10C* map within the minimal duplication interval and are maternally active. Although another imprinted, maternally expressed transcript (PIX1; UniGene Hs. 301667) has been identified in this region, it is noncoding and of unknown function. A paternally expressed antisense transcript was described at the *UBE3A* locus and was proposed to be involved in imprinted regulation of *UBE3A* [34]. A hypothetical inherited susceptibility could directly involve *UBE3A*, perhaps affecting expression or function, or indirectly affect imprinted repression on the paternal allele. For example, a relaxation of normal imprinted repression of the paternal allele would theoretically produce increased *UBE3A* expression in the brain analogous to that caused by interstitial duplication. Such a scenario might complicate efforts to detect parental-specific effects through genetic analysis of markers at this locus, and

is consistent with our data showing only marginally significant effects after stratification on parental-specific transmissions. Direct screening of *UBE3A* for functional coding mutations or variations in 10 autistic individuals did not identify significant changes [35]. Given the potential involvement of expression mechanisms, the potential functional effect of noncoding polymorphisms, and the relatively small number of subjects screened, the possible involvement of *UBE3A* variants in autism cannot be excluded.

Additionally, phenotypic overlap exists between AS and autism. AS-related maternal deletion of 15q11–q13 has been detected in patients diagnosed with autism [36]. AS is characterized by severe mental retardation, absent speech, seizure disorder, ataxia, stereotypical behaviors such as hand flapping, and unprovoked laughter [37]. Patients with interstitial duplications and inv dup(15) markers have been described as having features of AS (such as ataxia and/or poor motor coordination, seizures, mental retardation, and hand flapping) [14]. Although AS is related to deficiency of *UBE3A* in the brain and autism-related duplications would be expected to have copy-number-dependent overexpression of *UBE3A*, perturbations in either direction could theoretically produce deficits in functional domains in which UBE3A seems to have putative involvement, such as language, cognitive function, and neuronal excitability.

We were not able to replicate the findings of Cook and colleagues and of Martin, *et al*., who identified LD in *GABRB3* at markers *GABRB3* 155CA-2 and GABRB3, respectively [27,28]. We did find suggestive evidence of a paternal-specific effect at *D15S540* ($P = 0.0171$), although this nominal result is subject to possible type I error and did not remain significant after Bonferroni correction for testing multiple markers and sex-specific transmissions. *D15S540* is located ~ 250 kb downstream and centromeric from marker GABRB3, based on a published map of the region [31]. Similarly, the nominal result at *D15S156* ($P = 0.0448$) did not remain significant after correction. This marker is telomeric to the *GABRG3* and is very close to the peak linkage detected in another AD data set [22].

The importance, if any, of the genomic deletion at *D15S822* is unclear. Given the precedence for genomic deletions in this region, we sought to characterize this deletion using the hypothesis that it could have a functional effect or be in linkage disequilibrium with a susceptibility allele. Although a preliminary analysis of this deletion in a smaller set of families indicated a significantly increased frequency in autism families compared with that of controls, a more thorough analysis in a larger data set demonstrated only a nonsignificant trend toward increased frequency and the absence of significant association in those families carrying the deletion. It is possible that an unrecognized population stratification could obscure a relatively weak genetic effect, but it is not apparent that there is any likely functional effect of the deletion. *D15S822* is within a large (~ 200-kb) intron of *GABRG3*, and we have shown here that it is about 8 kb from a testis-specific gene of unknown function. Preliminary analysis of haplotype data for the *D15S822* deletion and flanking markers in the CLSA families indicates a common haplotype including marker alleles at *GABRA5* and *D15S156* in most individuals. If confirmed in other families, this could indicate that the deletion occurred relatively recently. Although it seems not to be significantly associated with autism in these families, it was important to document the deletion polymorphism, given its potential to complicate or distort genetic studies based on microsatellite markers. Without a PCR-based assay, the deletion is difficult to detect. Previously published data may be reevaluated at this marker using the assay described here.

Materials and Methods

Families. The sample for this study consisted of 94 families with at least two children with autism. In four families, the affected children were half-siblings. A detailed description of ascertainment and the demographics for this sample has been published [23]. All probands were at least 3 y of age and were clinically assessed with the Autism Diagnostic Interview–Revised [38] and the Autism Diagnostic Observation Schedule [39]. Probands were excluded from the study if they had a known medical or neurological condition suspected to be associated with their autistic phenotype (such as fragile X syndrome). All families provided written informed consent for participation in this study.

Molecular analysis. DNA was isolated from peripheral blood or lymphoblastoid cultures using the PureGene kit according to manufacturer's recommendations (Gentra Systems, Minneapolis, MN). The polymorphic microsatellite markers used here have been published and include *D15S817*, *D15S122*, *D15S210*, *GATA143C02* (*D15S1513*), *D15S540*, *D15S97*, *D15S822*, *D15S975*, *D15S156*, and *D15S219*; these markers are placed on the Marshfield genetic map and primer sequences are deposited in the Genome Database. Additional published markers corresponding to the *GABRB3* and *GABRA5* genes were used: GABRB3, *GABRB3* 155CA-2, *85CA* (*D15S511*), and *GABRA5* [40,41]. PCR used 5.5- or 10-μl reaction volumes containing 20 ng genomic DNA template and 0.6 μM primers and the Life Technologies Platinum *Taq* Master mix, according to the manufacturer's recommendations (Life Technologies, Gaithersberg, MD). Cycling conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 4 min. PCR products were separated by electrophoresis through 6% denaturing polyacrylamide sequencing gels, and images were obtained using a Hitachi FM BioII fluorescent laser scanner after gels were stained by SybrGold nucleic acid stain, according to manufacturer's recommendations (Molecular Probes, Eugene, OR).

A single-nucleotide polymorphism at the 5′ end of *GABRB3*, located 10 bp upstream of the transcription initiation site for exon 1a [42], was included in the markers analyzed in this study. The polymorphism was detected by single-strand conformational polymorphism analysis and subsequent DNA sequencing of PCR products for *GABRB3* exon 1a (Y.-h.C. and J.S.S., unpublished data). The *GABRB3* –10 C/G variant was assayed by digestion of PCR products with *Bst*UI, after amplification of genomic DNA with primers for exon 1a as described [43]. Digestion products were separated by electrophoresis through 10% non-denaturing polyacrylamide gels. The C allele corresponds to an undigested PCR product of 206 bp and the G allele appears as digested fragments of 178 and 28 bp.

Statistical analysis. Linkage disequilibrium analysis was done using the T_{SP} statistic [44]. This test allows the use of nuclear families with multiple affected individuals and evaluates all alleles of multiallelic markers. The sample in this analysis was 94 affected sib-pair families. As 13 markers were analyzed in this study, Bonferroni correction for the overall analysis at all markers led to an adjusted significance threshold of $P < 0.0039$. The Bonferroni correction is likely to be conservative, given that these markers are linked to each other. The overall data set was analyzed, and suggestive markers were stratified by parental-specific transmission to test the hypothesis that LD of a gene/marker could be related to imprinting. Analysis of *D15S122* included tabulation of overall and

parental-specific transmissions for each allele. The genotype at *D15S122* in CEPH individual 1331-01 is 145/147 bp. Families with half-siblings or with incomplete genotype data at a particular marker are considered "triads" in the T_{SP} analysis (Tables 1 and 2).

Linkage analysis was done for an interval defined by markers *D15S817* to *D15S118* (Fig. 1) using the Allegro package [45] to calculate MMLS/het scores. CRIMAP was used to determine sex-averaged and sex-specific genetic map distances between markers in the CLSA autism families and to compare the autism maps with maps developed from genotype data on 40 CEPH families [22]. The χ^2 value is 4.6[$Log_{10}Likelihood_{AD}$–$Log_{10}Likelihood_{CEPH}$], and degrees of freedom (*df*) are based on the number of marker distances tested (Fig. 2).

D15S822 deletion analysis. PAC DNA for RPCI-1 clone 69I9 was isolated by standard methods. Southern blot analysis of PAC DNA digested with *Pst*I used standard methods [46] and a probe corresponding to purified *D15S822* PCR product. A 4.8-kb *Pst*I fragment containing the *D15S822* microsatellite marker was subcloned from 69I9 into *Pst*I-digested pBluescript II SK– vector and sequenced using a combination of vector and custom sequencing primers, using ABI Big Dye terminator sequencing chemistry (PE Biosystems, Foster City, CA) in ABI 3700 or 377 sequencers in the Vanderbilt University Shared Sequencing Resource. Identification of *D15S822* sequence (GenBank acc. no. G07911) and location of restriction sites was determined by analysis with MacVector sequence analysis software (Genetics Computer Group, Madison, WI). The entire 4.8-kb fragment was hybridized to Southern blots containing *Pst*I-digested genomic DNA from controls and individuals with apparent null alleles at the marker locus. Repeat suppression conditions were used, in which denatured labeled probe was incubated for 30 min at 65°C in 150 μl of a 5 × SSC solution containing ~ 1 mg denatured, unlabelled, sheared human placental DNA before filter hybridization. Subsequent Southern blot analysis used a *Pst*I–*Eco*RV fragment (Fig. 3A) corresponding to one end of the *Pst*I fragment and nucleotide positions 63,734–64,961 in GenBank acc. no. AC006602. Primers for the duplex PCR screening assay to detect both deleted and non-deleted alleles at *D15S822* were: combination-63782, 5′–GGCATCTTAACTTGCTTCACTTTC–3′; deletion-58416, 5′–CAATCCTC-CAACTTTGTTCTTCTG–3′; and control-63179, 5′–GTCCTTTTTGGGAATA-CACCTTCA–3′. The number refers to the nucleotide position of the 5′ end in GenBank acc. no. AC006602, and the combination, deletion, or control designations are shown in Fig. 3B. Product sizes are 604 bp for a non-deletion chromosome and 429 bp for a deletion chromosome (Fig. 3C). A hypothetical PCR product of 5.4 kb corresponding to the combination and deletion-specific primers, amplifying from non-deletion alleles, is not found with standard PCR conditions. The duplex screening PCR assay used 20 ng DNA template in 20-μl PCR reactions essentially as described above but with 1 μM primers and an annealing temperature of 61°C. Reaction products were separated by electrophoresis through 2% NuSieve (3:1) agarose gels (FMC, Rockland, ME) and images were obtained after ethidium bromide staining. The sample for this analysis consisted of 158 CLSA sib-pair and trio families, 185 families from the Duke University group (88 simplex, 79 multiplex, and 18 unconfirmed multiplex), and 190 families from the Greenwood Genetic group (174 simplex and 16 multiplex).

For statistical analysis of the deletion, we compared the frequency in unrelated chromosomes from autism families and non-autism Caucasian controls using Fisher's exact test. We analyzed transmission of the *D15S822* deletion by comparing observed versus expected transmission from paternal or maternal carriers in the proband of each family, followed by Fisher's exact test. Analysis for association within the families carrying the deletion was made by examining all possible transmissions to affected and unaffected individuals using the pedigree disequilibrium test statistic [47].

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