into an SCF complex facilitates activation of Cdc34's intrinsic conjugating activity. Rbx1 may also function as a ubiquitin carrier, a possibility that remains to be tested.

Rbx1 is a highly conserved protein (*15*) that plays an essential role in SCF function in part by recruiting Cdc34. Consistent with this, *rbx1* mutants have been independently found that are synthetically lethal with *cdc34* mutants (*22*). The closest Rbx1 homolog in yeast is the essential anaphase-promoting complex (APC) subunit Apc11 (*23*), which may act in a parallel fashion, binding to the Cdc53 homolog Apc2 and recruiting an E2 into the APC, making the APC a distantly related member of the SCF family of E3s. Sequences related to the R-box motif in Rbx1 can be found in several other *S. cerevisiae* proteins associated with ubiquitination, including Hrd1, Rad18, and Ubr1, which have been implicated in 3-hydroxy-3-methylglutary–coenzyme A reductase ubiquitination (*24*), DNA repair (*25*), and the N-end rule pathway (*26*), respectively, and in four uncharacterized open reading frames (Fig. 4F). Rad18 and Ubr1 form independent complexes with the E2 Rad6 (*27–29*), which controls the N-end rule pathway, DNA repair, and telomeric silencing (*25–27*). Most R-box proteins are much larger than Rbx1 and may themselves contain substrate recognition domains, as has been demonstrated for Ubr1 (*29*). In plants, the Prt1 R-box protein is genetically implicated in the N-end rule pathway (*30*). In mammals, Mdm2, a p53 E3, also contains an R-box–related motif (*14*) that is required for its function (*31*). Currently, ubiquitin-carrying HECT-domain proteins are the only well characterized class of E3s not linked to R-box proteins. Thus, E3s may fall into two main classes: the SCF class that require R-box proteins and the HECT class that do not. The finding that mammalian Rbx1 is a component of the von Hippel–Lindau (VHL)–Elongin B/C–Cul2 complex (*15*) suggests that these complexes may also be involved in the transfer of ubiquitin or ubiquitin-like proteins.

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13. Yeast extracts from isogenic YM4575 (*GRR1*) or YM4576 (*grr1Δ*) strains (*32*) or CB018 (*pep4::HIS3*) were fractionated on DEAE-Sephacryl (*33*). Proteins eluting with 250 and 500 mM KCl were collected (F250 and F500, respectively). Immunoblot analysis revealed Cdc34 only in the F500 fraction. Twenty-microliter ubiquitination reactions (30 to 60 min, 25°C) contained 500 nM Cdc34, 300 nM E1, 2 mM adenosine triphosphate (ATP), an ATP-regenerating system, 20 μM ubiquitin, 0 to 120 μg of F250, and 50 ng of active or kinase-defective (K-) HA-Cln1/GST-Cdc28 (*12*). Cln1 autophosphorylation was performed at 25°C in the presence of 1 mM ATP for 30 min. Reaction mixtures were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and products were visualized by immunoblotting with ECL detection (Amersham). SCF complexes were purified from insect cells 40 hours after infection with antibodies to tagged proteins (*12*). A baculovirus encoding untagged Grr1 (in pVL1392) was produced with Baculogold (Pharmingen). Rabbit anti-Grr1 was generated with the peptide MDQDNNNHND-SNRL (see legend to Fig. 4 for amino acid abbreviations). *RBX1* and *rbx1-1* (*16*) extracts were prepared from cells shifted to 38°C for 16 hours. Strains (*MATa RBX1::HIS3 ura3 leu2 trp1 lys2 his3Δ200 can1-100 cyh2*) were maintained by a plasmid containing either a wild-type (pDK101) or mutant (pDK102) version of *RBX1* in pRS314.
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16. *RBX1* was disrupted by inserting the *LEU2* gene into the Nsi I site in the *RBX1* open reading frame in pDK100 (containing a genomic *RBX1* fragment in pRS316). A Sac I–Sph I fragment from pDK100 was used to transform Y80 (*MATa can1-100 ade2-1 his3-11, 15 leu 2-3, 112 trp1-1 ura3-1*) carrying pDK100 to generate the disruption strain. Strains that could not grow without pDK100 were chosen, and integration of the *LEU2* gene was confirmed by Southern (DNA) blot analysis. The *rbx1-1* allele was generated by polymerase chain reaction mutagenesis (*34*) with primers flanking the open reading frame. The plasmid was rescued from a temperature-sensitive strain, retested, and sequenced.
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18. Baculoviruses encoding Rbx1 and MYC3-Rbx1 [in pVL1392 or pHI-100 by universal plasmid subcloning (*35*), respectively] were generated with Baculogold (Pharmingen).
19. For SCF assembly, we coinfected Hi5 cells (2×10^6) with recombinant baculoviruses. Forty hours later, cells were lysed in 50 mM tris-HCl (pH 7.5) containing 100 mM NaCl, 0.5% NP-40, 2 mM dithiothreitol, 0.1 mM $Zn(OAC)_2$, and protease inhibitors (Boehringer-Mannheim). Cleared lysates were immunoprecipitated for 1 to 2 hours with 10 μl of beads containing immobilized antibodies. Proteins were separated by SDS-PAGE and immunoblotted. HA-Cln1 and Cdc34 ubiquitination was performed at 25°C with 10 μl of agarose beads containing SCF complexes with or without Rbx1 supplemented with 500 nM Cdc34, 300 nM E1, 4 mM ATP, 20 μM ubiquitin (or 40 μM GST-Ub^{RA}), and 50 ng of HA-Cln1/GST-Cdc28. Reaction mixtures were separated by SDS-PAGE and immunoblotted.
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Male Homosexuality: Absence of Linkage to Microsatellite Markers at Xq28

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Several lines of evidence have implicated genetic factors in homosexuality. The most compelling observation has been the report of genetic linkage of male homosexuality to microsatellite markers on the X chromosome. This observation warranted further study and confirmation. Sharing of alleles at position Xq28 was studied in 52 gay male sibling pairs from Canadian families. Four markers at Xq28 were analyzed (DXS1113, BGN, Factor 8, and DXS1108). Allele and haplotype sharing for these markers was not increased over expectation. These results do not support an X-linked gene underlying male homosexuality.

Previous studies have suggested that there is a genetic component in male sexual orientation. These include controlled family studies that have shown an increased frequency of homosexual brothers of homosexual index subjects as compared to heterosexual index subjects (*1*, *2*) and twin studies, which have shown increased concordance for homosexu-

Table 1. Allele sharing by state in 52 homosexual brother pairs.

Marker	Hetero-zygosity	Sharing		Nonsharing		Chi square
		Observed	Expected	Observed	Expected	
DXS1113	0.7	34	33.80	18	18.20	0.00
BGN	0.82	30	30.68	22	21.32	0.04
Factor 8	0.68	35	34.32	17	17.68	0.04
DXS1108	0.74	32	32.76	20	19.24	0.05
Last three loci combined	0.99	24	26.96	28	25.61	0.44

al orientation in monozygotic as compared to dizygotic twins (*3*). On the other hand, the similar rates of male homosexuality in biological and adoptive siblings of male homosexual index subjects (*3*), coupled with methodological uncertainties in family and twin studies of homosexuality, suggest caution in accepting a genetic-epidemiological basis for homosexuality (*4*, *5*).

The strongest support for a genetic component in male sexual orientation came from the studies of Hamer *et al.* (*6*, *7*), who posited the involvement of an X-linked gene at position Xq28, based on family recurrence patterns and molecular analysis of the X chromosome in sibships in which there were multiple brothers with homosexual orientation. Specifically, Hamer and colleagues obtained family history information from 76 gay male index subjects and 40 gay brother pairs about the sexual orientation of the first-, second-, and third-degree relatives, with follow-up interviews of a smaller proportion of relatives. They reported increased rates of homosexual orientation in the maternal uncles and male cousins through maternal aunts, which was suggestive of X-linked inheritance. Molecular analysis of the X chromosome revealed an excess of allele sharing in the region of Xq28 in 40 homosexual brother pairs (*6*) and, to a lesser extent, in a follow-up study of 33 additional pairs (*7*).

However, the evidence for X linkage has been questioned on theoretical and empirical grounds (*8*, *9*). Most would agree that male homosexual orientation is not a simple Mendelian trait. There would be strong selective pressures against such a gene. Hamer's identification of a contribution from a gene near Xq28 to homosexuality in some families that were selected for X-linked transmission of that trait might be fraught with type 1 (false positive) error. This is important to consider, given the irreproducibility of many linkage reports for complex behavioral traits.

Given the political and social ramifications of gene linkage in homosexuality, we launched independent genetic studies of male sexual orientation in Canada. Specifically, we advertised in Canadian gay news magazines (*Xtra* and *Fugue*) for families in which there were at least two gay brothers. One hundred and eighty-two individuals responded to the advertisement. The respondents volunteered information about the sexual orientation of individuals in their families, including siblings, parents, uncles, aunts, and first cousins, although all members of the extended family were not directly interviewed. The 182 families included 614 brothers, 269 (44%) of whom were homosexual. There were 148 families with two gay sons, 34 families with three, and two families with four. The high rate of sibling concordance reflects the nature of the advertisement. The sample included 270 sisters, 49 (18%) of whom were said to be gay. This rate is high compared to the frequency of homosexual orientation in women as ascertained in most population-based studies, which suggest a sister concordance rate of 14% (*10*).

Our molecular analysis was based on 52 gay sibling pairs from 48 families who were willing to provide blood samples. Sexual orientation was confirmed for all subjects at the time of blood sampling by the direct questioning of a gay interviewer. The index subject read gay magazines and volunteered that he was gay, and this observation was corroborated by interviewing the gay brother. We believe that the rate of false positives, as in Hamer's study, was low (*6*). The sample included 46 families with two gay brothers. There were two families with three gay brothers, and these were considered as six pairs. Four markers were analyzed (DXS1113, BGN, Factor 8, and DXS1108), along a 12.5-centimorgan (cM) region of Xq28. The methods were as described (*11*). The allelic sharing is shown in Table 1.

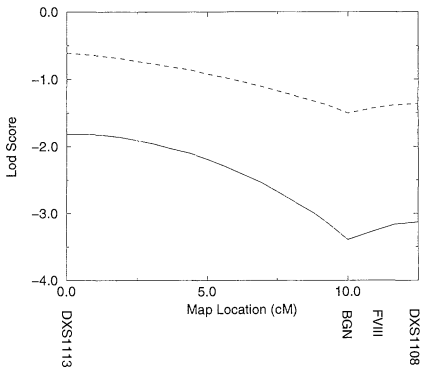

Fig. 1. Multipoint map for Xq28. Multipoint lod scores were calculated along the 12.5-cM region for two values of λ_s (2.0, solid line; 1.5, dashed line), where λ_s is the ratio for homosexual orientation in the brothers of a gay index subject, as compared to the population frequency, that is attributable to a gene in this region. Very strong exclusion is obtained for lod scores <-2.0, and strong exclusion is obtained for lod scores <-1.0.

Genotyping was performed on DNA samples from the brothers themselves without genotyping of parents (alleles were identified by comparison with population-based controls, known as "identical by state," rather than by confirmation of maternal transmission, known as "identical by descent"). Maternal DNA was difficult to obtain. As controls, we included an additional 33 sibling pairs who were concordant for multiple sclerosis. These were genotyped simultaneously with the gay sibships (Table 2). Allele scoring was performed independently by two evaluators who were blind to the status of the sibship.

A priori, a pair of brothers will share an X-linked maternal allele, identical by descent with probability = 1/2. Therefore, for a marker with heterozygosity H, under the null hypothesis of no excess sharing, a brother pair will share an allele identical by state with probability $1/2 + 1/2\,(1 - H) = 1 - H/2$. For an X-linked trait-influencing locus in the region, the sharing will be increased. For the distal three markers taken as a haplotype, the probability that brothers share the full haplotype is approximately $(1 - H/2)\,(1 - \theta)^2$, where θ is the recombination fraction in the entire interval (2.5% for all three markers), and H is the heterozygosity of the full haplotype (which is 1 minus the product of the homozygosities at the three loci, assuming linkage equilibrium).

Table 1 shows no excess sharing for any of the four markers tested nor for the haplotype of the distal three loci. These results are not consistent with an X-linked gene underlying sexual orientation in this region of the X chromosome.

We further analyzed these data with mul-

Table 2. Allele sharing in 33 control brother pairs.

Marker	Hetero-zygosity	Observed sharing	Expected sharing	Chi square
DXS1113	0.7	23	21.45	0.34
BGN	0.82	22	19.47	0.80
Factor 8	0.68	20	21.78	0.43
DXS1108	0.74	23	20.79	0.63

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tipoint sib pair analysis by means of the computer program ASPEX (*12*). Multipoint lod (logarithm of the odds ratio for linkage) scores were calculated along the 12.5-cM region for two values of λ_s (2.0 and 1.5), where λ_s is the ratio for homosexual orientation in the brothers of a gay index subject, as compared to the population frequency, that is attributable to a gene in this region. Very strong exclusion is obtained for lod scores <-2.0, and strong exclusion is obtained for lod scores <-1.0 (*13*). As depicted in Fig. 1, λ_s values of two or greater can be very strongly excluded. Values of 1.5 or greater can also be strongly excluded. The lod scores were clearly negative for all values of λ_s .

Hamer and colleagues described linkage of male homosexuality to polymorphic markers at Xq28 in 40 brother pairs. The sharing was 33/40, deemed to be significant with a λ_s value of 2.86 (*6*). In a follow-up study of 33 gay male sibling pairs (32 informative), 22 shared all the Xq28 markers (*7*). Our sample comprised 46 sib pairs and 2 sib trios. The sharing of distal Xq28 markers in the 46 sib pairs was 20/46. For one of the sib trios, all three brothers shared the same X chromosome; for the other trio, two shared the same X chromosome and the other was different. Therefore, forming independent sib pairs by picking two pairs out of three for each sibship gives a total X-chromosome sharing of either 2 out of 4 or 3 out of 4. For comparison with the results of Hamer *et al.*, we used the more favorable 3 out of 4, which gave a total of 23 out of 50 chromosomes shared for our sample. This result was highly different statistically from the first study of Hamer (*6*) (chi square value = 11.09, $P < 0.001$) but not statistically different from the second study (*7*) (chi square = 3.21, $P > 0.05$). Combining the two replication studies gives a total sharing of 45 out of 82 (55%), which was not significantly different from 50% (chi square = 0.78, $P > 0.30$). Also, the sharing for the two replication studies combined is significantly different from the original study of Hamer *et al.* (chi square = 7.74, $P < 0.01$).

It is unclear from the original study to what extent families were excluded to produce the data set in which the positive linkage analysis was reported. Families were excluded if a father was gay or if there were any first-degree lesbian relatives. By these precise criteria, two sib pairs would have been excluded from our study (one with a gay father and one with two gay parents). For the remaining pairs, the linkage evidence was the same as for the entire group.

It is unclear why our results are so discrepant from Hamer's original study (*6*). Because our study was larger than that of Hamer *et al.*, we certainly had adequate power to detect a genetic effect as large as was reported in that study. Nonetheless, our data do not support the presence of a gene of large effect influencing sexual orientation at position Xq28.

Although we found no evidence of linkage of sexual orientation to Xq28, these results do not preclude the possibility of detectable gene effects elsewhere in the genome.

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