Retroposition of autosomal mRNA yielded testis-specific gene family on human Y chromosome

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Most genes in the human NRY (non-recombining portion of the Y chromosome) can be assigned to one of two groups: X-homologous genes or testis-specific gene families with no obvious X-chromosomal homologues[1,2]. The *CDY* genes have been localized to the human Y chromosome[1], and we report here that they are derivatives of a conventional single-copy gene, *CDYL* (*CDY*-like), located on human chromosome 13 and mouse chromosome 6. *CDY* genes retain *CDYL* exonic sequences but lack its introns. In mice, whose evolutionary lineage diverged before the appearance of the Y-linked derivatives, the autosomal *Cdyl* gene produces two transcripts; one is expressed ubiquitously and the other is expressed in testes only. In humans, autosomal *CDYL* produces only the ubiquitous transcript; the testis-specific transcript is the province of the Y-borne *CDY* genes. Our data indicate that *CDY* genes arose during primate evolution by retroposition of a *CDYL* mRNA and amplification of the retroposed gene. Retroposition contributed to the gene content of the human Y chromosome, together with two other molecular evolutionary processes: persistence of a subset of genes shared with the X chromosome[3,4] and transposition of genomic DNA harbouring intact transcription units[5].

We had previously identified a single full-length cDNA clone from *CDY* but had mapped homologous sequences to two different locations on the human Y chromosome[1]. We explored the possibility that there might be multiple functional *CDY* genes by isolat-

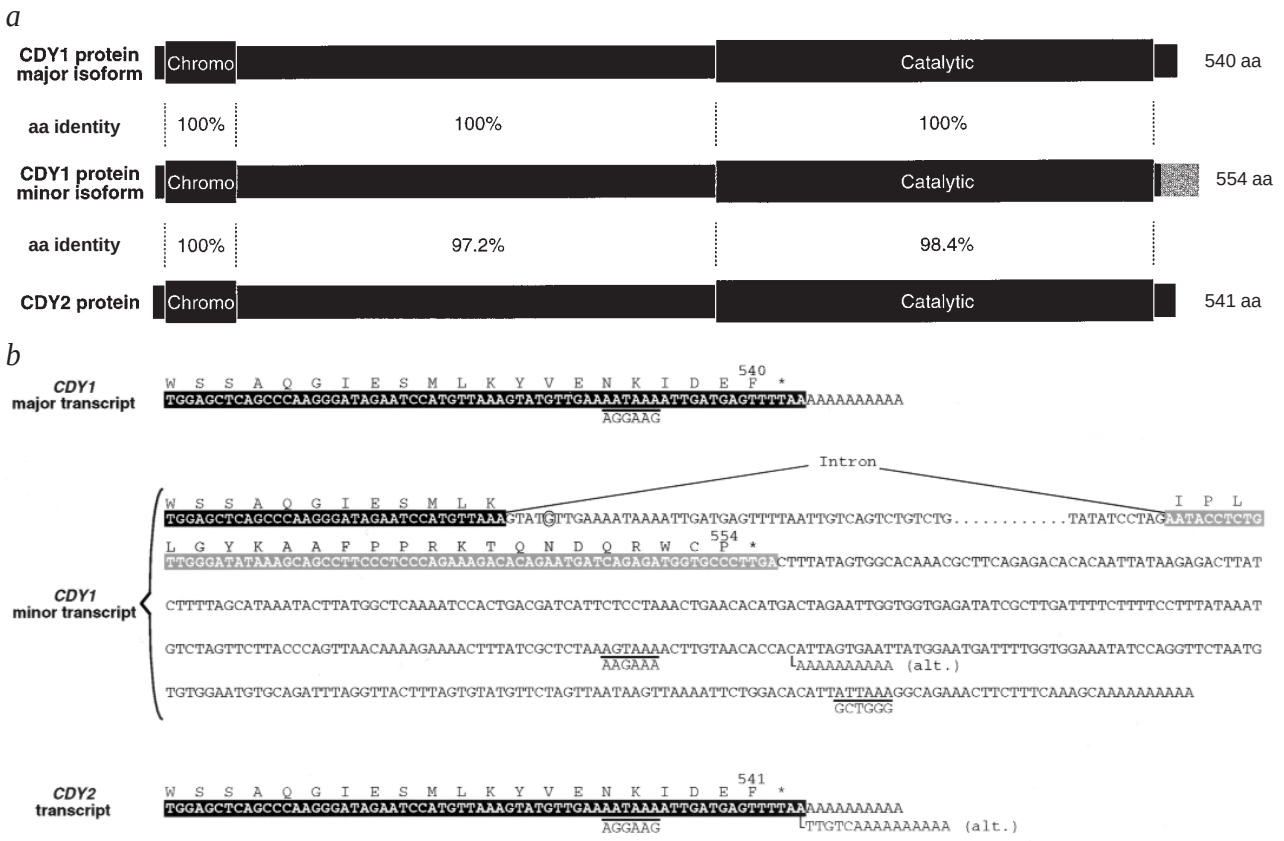

Fig. 1 Human CDY proteins and transcripts. ***a***, Schematic representation of predicted CDY1 and CDY2 proteins. Positions of chromo and putative catalytic domains[1] are indicated, as are protein lengths in amino acid residues. CDY1 major and minor isoforms differ only near their C termini (grey bar in C region of CDY1 minor isoform). ***b***, 3′ portions of *CDY1* (major and minor) and *CDY2* transcripts. Shown are partial cDNA and predicted amino acid sequences; coding sequence is placed against a black background (grey in the case of differential portion of *CDY1* minor transcript); terminal residues of predicted proteins are numbered. Part of the intron (genomic) sequence of the *CDY1* minor transcript is shown; this intron evolved from what had been exon 9 of *CDYL* (Fig. 2). The fifth nucleotide of this intron is circled; the appearance of a G at this position (where 84% of introns[19] have a G, but where human *CDYL* and mouse *Cdyl* have a T) may have contributed to the evolution of this novel intron. Alternative poly(A) tracts observed in some *CDY1* minor and *CDY2* cDNA clones are indicated. Below polyadenylation signals (underlined) are human *CDYL* sequences at corresponding positions; polyadenylation sites employed by human *CDY* genes apparently arose only after retroposition, through mutations that generated appropriate signal sequences.

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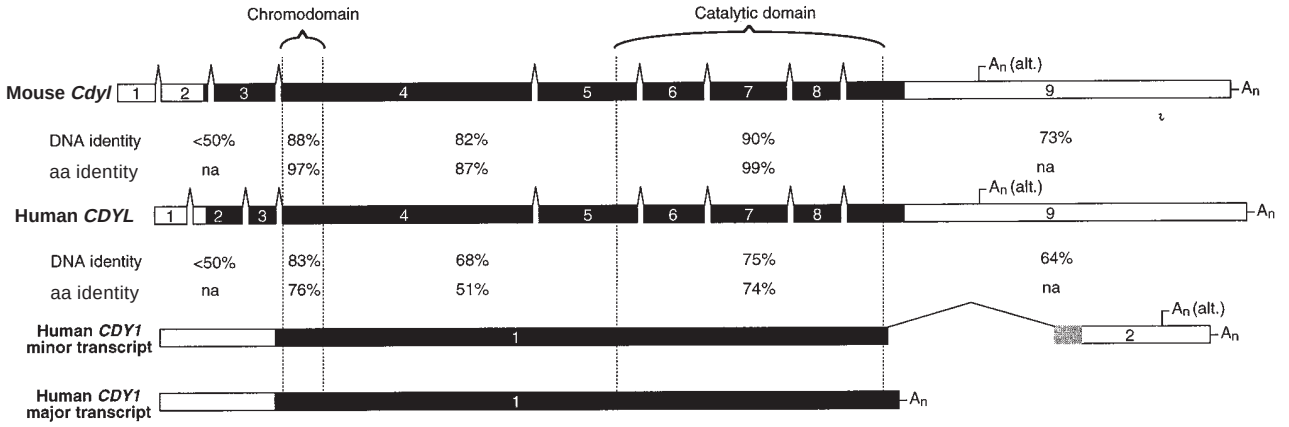

Fig. 2 Comparison of transcripts and encoded proteins from mouse *Cdyl*, human *CDYL* and human *CDY1* genes. Exons are numbered; positions of introns are indicated. Black and grey bars depict translated regions. Percentage identities are shown in several regions for both DNA and predicted amino acid sequences; na, not applicable. Note: 64% nucleotide identity was observed between the 3′ portion of human *CDYL* transcript and the intron/second exon of human *CDY1* minor transcript. Sequence similarity between *CDY1* genomic locus and *CDYL* falls abruptly (to less than 50% nucleotide identity) at both the 5′ and 3′ ends: immediately 5′ of *CDYL* exon 4, and 3′ of the polyadenylation site of *CDY1* minor transcript. Poly(A) tracts, including alternative locations observed in some cDNA clones, are indicated.

ing 25 additional cDNA clones from a human testis library. Sequence analysis of the 25 clones revealed two distinct species, which we designated *CDY1* (17 clones) and *CDY2* (8 clones). The sequence of *CDY1* was as previously reported[1]. Like *CDY1*, *CDY2* appears to encode a protein with a combination of chromatin-binding and catalytic domains (Fig. 1*a*). The predicted coding regions of *CDY1* and *CDY2* were 99% identical in nucleotide sequence, and the amino acid sequences of the predicted proteins were 98% identical. We observed an alternative 3′ region in 4 of 17 *CDY1* cDNA clones. Most of the putative protein encoded by this minor *CDY1* transcript is identical to that encoded by the major transcript; its carboxy terminus is divergent (Fig. 1*a*,*b*).

We had previously localized *CDY*-homologous sequences to Y chromosome deletion intervals 5L and 6F (ref. 1), but it was not apparent which of these map locations corresponded to which gene. Exploiting sequence differences between *CDY1* and *CDY2*, we designed PCR assays specific to each of the two genes. Using genomic DNAs from individuals carrying partial Y chromosomes as templates[6], we mapped *CDY1* to interval 6F, and *CDY2* to interval 5L (data not shown). We conclude that at least two distinct functional *CDY* genes exist on the human Y chromosome, and that they encode protein isoforms. We cannot exclude the possibility that there are multiple *CDY1* genes in interval 6F, multiple *CDY2* genes in interval 5L, or additional *CDY* species that we failed to identify.

To determine the intron/exon structures of *CDY1* and *CDY2*, we partially sequenced genomic BAC clones corresponding to each of the genes. *CDY1* and *CDY2* genomic sequences were found to be collinear with the major *CDY1* and *CDY2* transcripts as captured in cDNA clones, with no evidence that any intronic sequences had been excised. In the case of the minor *CDY1* transcript, it appeared that a single intron had been excised; the splice donor site was located within, and near the 3′ end of, the single exon of the major transcript (Fig. 1*b*). Further evidence of the atypical nature of *CDY* genes arose from examining their polyadenylation sites. For 13 of 17 *CDY1* and 5 of 8 *CDY2* cDNA clones, the poly(A) tract was located immediately 3′ of the predicted stop codon (Fig. 1*b*). *CDY1* and *CDY2* may be the only mammalian nuclear genes in which polyadenylation is known to occur so close to the stop codon.

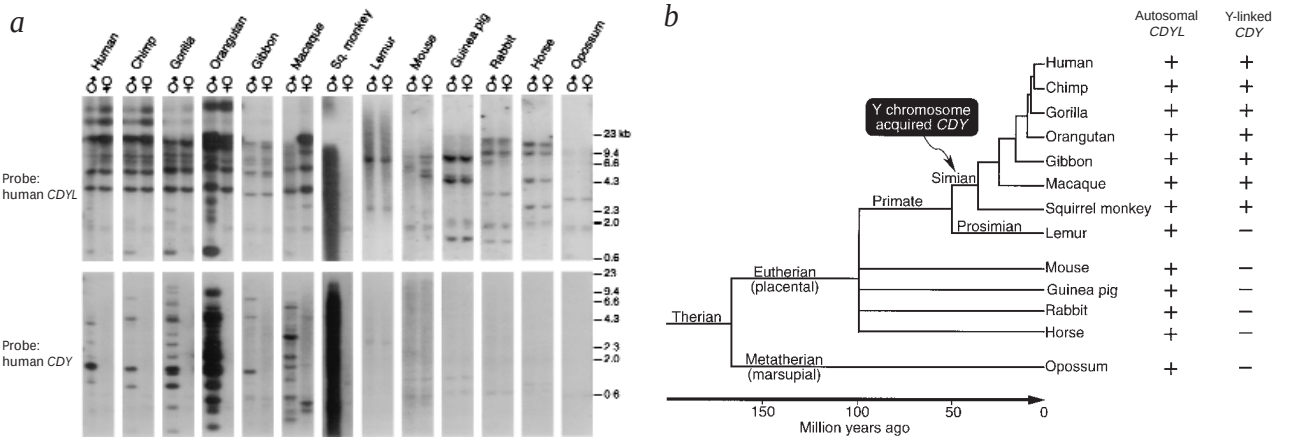

Fig. 3 Homologues of *CDYL* and *CDY* in diverse mammalian species. ***a***, Southern blots of male and female genomic DNAs from 13 mammalian species hybridized with cDNA fragments corresponding to the entire coding sequence of either human *CDYL* (top, *Taq*I digest) or human *CDY1* (bottom, *Bgl*II digest). At low stringency, *CDYL* probe hybridized with male-female common (presumably autosomal) homologues in all 13 species. It also cross-hybridized with Y-encoded homologues in some primates, especially orangutan and squirrel monkey. *CDY* probe identified Y-encoded homologues in all primates except lemurs. It also cross-hybridized weakly to the autosomal homologue in some species. Positions of size markers are shown at right. The apparent smears in the squirrel monkey lanes are composed of intense, discrete fragments, as revealed by shorter exposures. ***b***, Summary and interpretation of Southern-blot data. The 13 species tested are arranged phylogenetically[20–22].

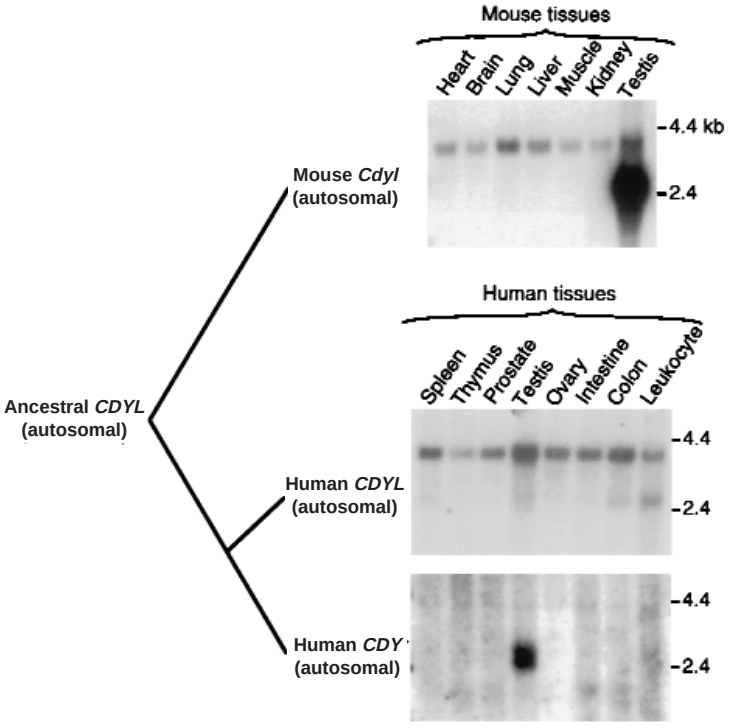

Fig. 4 Tissue distributions of mouse *Cdyl*, human *CDYL* and human *CDY* transcripts. Northern blots incubated cDNA fragments corresponding to the entire coding sequence of either mouse *Cdyl* (top), human *CDYL* (middle), or human *CDY1* (bottom) reveal tissue expression patterns; the phylogenic relationship of these genes (illustrated on left) is based on data presented in Fig. 3.

To address the evolutionary origin of *CDY* on the human Y chromosome, we sought homologues that might exist elsewhere in the genomes of humans or mice. We identified an autosomal homologue in both humans and mice by incubating a *CDY*-derived probe, at low stringency, with testis cDNA libraries from the two species. We will refer to the human and mouse autosomal homologues as *CDYL* and *Cdyl*, respectively. We determined the sequence of the human *CDYL* transcript by merging the sequences of four incomplete but substantially overlapping cDNA clones; we found no sequence differences among the four cDNA clones in regions of overlap. Similarly, we derived the sequence of mouse *Cdyl* by merging the sequences of ten incomplete but overlapping cDNA clones. In both species, the autosomal transcript, similar to human *CDY*, appears to encode a protein with an amino-terminal chromo domain and a C-terminal catalytic domain (Fig. 2). The predicted mouse and human CDYL proteins have 93% amino acid identity overall, with even greater similarity evident in the chromo and catalytic domains (Fig. 2). In contrast, the predicted human CDYL and CDY proteins have only 63% amino acid identity; again, greater similarity is evident in the chromo and catalytic domains (Fig. 2). These results suggest that human *CDYL* and mouse *Cdyl* are orthologues, and human *CDYL* and *CDY* are paralogues. They also suggest that evolutionary constraints operating on the chromo and catalytic domains of the CDYL and CDY proteins account for the relatively high amino acid identities observed there.

Chromosomal mapping of the human *CDYL* and mouse *Cdyl* genes indicate that they are indeed orthologues. In humans, we localized *CDYL* to the distal short arm of chromosome 6 (distal to marker NIB1876 and proximal to WI-4489) by radiation hybrid mapping. In mice, we localized *Cdyl* to the proximal portion of chromosome 13 (distal to *Fim1* and proximal to *D13Mit18*) by meiotic linkage mapping. These portions of human chromosome 6 and mouse chromosome 13 were previously shown to be syntenic[7]. We conclude that a gene much like human *CDYL* and mouse *Cdyl* existed in the common ancestors of mice and humans.

Our experiments to this point left another evolutionary question unanswered: which came first, *CDY* or *CDYL*? The first indication came from studies in mice, in which we were unable to identify a Y-linked homologue of human *CDY* by either Southern-blot analysis (Fig. 3*a*) or screening of testis cDNA libraries. These findings are consistent with *CDYL* having given rise to *CDY* sometime after divergence of the mouse and human lineages.

Additional evidence that *CDYL* gave rise to *CDY* came from analysis of the human *CDYL* and mouse *Cdyl* gene structures. We found that each of these autosomal genes contains eight introns (Fig. 2). As one would expect for orthologues, most introns were positioned at corresponding sites in human *CDYL* and mouse *Cdyl*. The presence of numerous introns in the *CDYL*/*Cdyl* orthologues, contrasted with the paucity of introns in the human *CDY* genes, led us to consider whether *CDY* had arisen by reverse transcription of a spliced *CDYL* mRNA, with subsequent integration into the Y chromosome.

Closer examination of the sequence data corroborated this retroposition model. Excluding a few hundred nucleotides at the 5′ terminus, the nucleotide sequence of the human *CDY1* (or *CDY2*) genomic locus is essentially collinear with that of the mature human *CDYL* (or mouse *Cdyl*) transcripts. Even the single intron of the minor *CDY1* transcript is collinear with, and homologous to, *CDYL* exon 9 (Fig. 2). Thus, with respect to evolutionary origins, all but the most 5′ portions of human *CDY1* and *CDY2* genomic loci can be accounted for by mature *CDYL*/*Cdyl* transcripts. These findings are as one would predict if *CDYL*/*Cdyl* intronic sequences had been excised before retroposition.

How long ago did the retroposition event occur? To address this question, we incubated probes derived from human *CDYL* and *CDY* with Southern blots of male and female genomic DNAs from various mammalian species. Two conclusions emerged from this analysis (Fig. 3*a*): (i) the autosomal (male-female common) *CDYL* gene appears to be widely conserved among the mammals tested, including opossum, a marsupial; and (ii), the Y-chromosomal (male-specific) *CDY* family appears to be restricted to simian primates, including apes, Old World monkeys (for example macaques) and New World monkeys (for example squirrel monkeys, whose sequences appear to have undergone extraordinary amplification on the Y). We observed no male-specific hybridization fragments in prosimians (lemurs) or any of the non-primate mammals tested. The simplest interpretation of these findings is that the retroposition event occurred in the simian lineage after its divergence from prosimians but before the split between Old and New World monkeys, perhaps 40–50 million years ago (Fig. 3*b*). Given the age of the retroposed *CDY* genes, it is not unexpected that certain hallmarks of retroposition are absent: we found no evidence of target site duplication or of a poly(A) tract in *CDY* genomic sequences. The observed 99% identity between the coding sequences of human *CDY1* and *CDY2* (Fig. 1*a*; as contrasted with 73% identity between human *CDY* and human *CDYL*) suggests that their coexistence is the result of subsequent amplification on the Y chromosome.

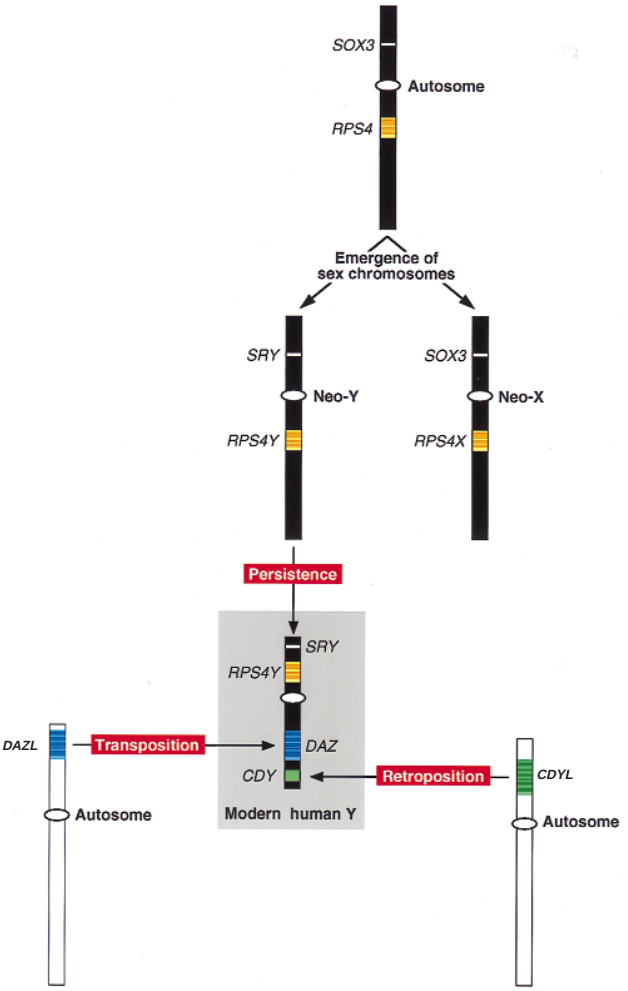

Fig. 5 Schematic representation of three molecular evolutionary processes that contributed genes to human NRY. Persistence (top): an autosomal pair gave rise to the neo-Y and neo-X (subsequently enlarged by fusion with other autosomes or autosomal segments; data not shown; refs 23,24). *SRY*, *RPS4Y* and several other genes derived from these ancestral autosomes persist as X-homologous genes in the human NRY (refs 1–4,25). Transposition: the *DAZ* genes arose by transposition (and subsequent amplification; data not shown) of autosomal genomic DNA containing the entire *DAZL* transcription unit[5]. Retroposition: the *CDY* genes arose by integration (and subsequent amplification; data not shown) of a reverse-transcribed copy of a processed mRNA derived from the autosomal *CDYL* gene. Gene sizes are not to scale.

Do all members of the *CDY*/*CDYL* gene family serve male-specific functions? We had previously demonstrated by northern-blot analysis that human *CDY* genes are abundantly transcribed in adult testis but are not detectable in other adult tissues[1] (Fig. 4). In contrast, human *CDYL* appears to be expressed at a modest and comparable level in all tissues examined (Fig. 4), indicating that it may perform a housekeeping function. In mice, *Cdyl* is characterized by two transcripts: (i) a ubiquitous one that is similar in length to the ubiquitous transcript of human *CDYL*; and (ii) a testis-specific one that is similar in length to the testis-specific transcript of human *CDY* (Fig. 4). These expression studies in humans and mice suggest that a genomic partitioning of housekeeping and testis-specific functions evolved in primates after retroposition. We interpret the mouse, which possesses no *CDY* homologue on its Y chromosome, as representing the evolutionarily ancestral condition. In humans, the autosomal gene *CDYL* appears to retain the housekeeping function but relinquished the testis-specific function, the latter of which seems to have transferred to the Y-linked *CDY* genes.

We propose, on the basis of these and other findings, that the gene content of the human NRY was forged during evolution through the interplay of three molecular processes: retroposition, transposition and persistence (Fig. 5). In certain respects, the process of *CDY* retroposition is reminiscent of the transposition process by which the *DAZ* gene family arose on the Y chromosome[5]. The net result of both evolutionary processes was the duplicative transfer of an autosomal gene to the Y chromosome, with retention of the progenitor autosomal gene. Subsequent to retroposition (as exemplified by *CDY*) and transposition (as exemplified by *DAZ*) the genes transferred to the Y chromosome were therein amplified. The expression pattern of the Y-linked *CDY* genes, however, is very different to that of their autosomal progenitor, *Cdyl*/*CDYL* (Fig. 4), whereas expression of the Y-linked *DAZ* genes is quite similar to that of their autosomal progenitor, *DAZL* (also known as *DAZLA*, *DAZH* or *SPGYLA*; refs 5,8–11). In the case of *CDY*, the nascent Y-linked gene was fashioned from a fully processed, reverse-transcribed cDNA which, we speculate, fortuitously integrated near an existing promoter or into an otherwise transcriptionally permissive locale on the Y chromosome. In contrast, in the case of *DAZ*, a segment of genomic DNA containing an entire transcription unit was duplicated from an autosome onto the Y chromosome[5]. The transposed genomic DNA of *DAZ* probably included not only introns and exons but also the promoter of the ancestral gene.

Retroposition has long been suspected to figure prominently in the evolution of animal Y chromosomes—but solely as a means of marking the decay of genes previously shared with the X chromosome. In *Drosophila melanogaster*, molecular studies have suggested that insertional mutagenesis via retroposition was a major driving force in Y gene decay[12–14]. An accumulation of retroposed elements on the mouse Y chromosome[15] suggested that this paradigm might extend to mammals[16]. Thus, it is reasonable to speculate that retroposition—especially of highly transposable elements—may have had an important role in Y gene decay in many animal lineages. As illustrated here by *CDY*, however, retroposition has also provided a mechanism for gene building during the evolution of the human Y chromosome.

Methods

Identification of cDNA clones. The cDNA insert of plasmid pDP1660 contains the entire ORF of human *CDY1*, as previously reported[1]. To identify additional *CDY* clones, we labelled the plasmid insert with ^{32}P-dCTP by random priming and used this probe to screen a human adult testis cDNA library (Clontech). We incubated blots overnight at 65 °C in Na_iPO_4 (0.5 M), 7% SDS, followed by three washes of 15 min each at 65 °C in 0.1×SSC, 0.1% SDS. We identified human *CDYL* clones by rescreening the same library with the same probe at lower stringency (incubation at 60 °C, as above; washing at 55 °C in 0.1×SSC, 0.1% SDS). We also used this probe and conditions to screen a mouse adult testis cDNA library (Clontech), resulting in the identification of mouse *Cdyl* clones.

PCR analysis of human genomic DNAs. We tested human genomic DNAs for the presence or absence of *CDY1* and *CDY2* using PCR assays specific to each of the genes. PCR primers for *CDY1* were 5′–GGCGAAAGCTGACAGCAA–3′ and 5′–GGGTGAAAGTTCCAGTCAA–3′. PCR primers for *CDY2* were 5′–GACCACAAGAAAACTGTGAG–3′ and 5′–GATCTGCTGCAATAGGGTC–3′. Thermocycling conditions were 30 cycles of 1 min at 94 °C, 45 s at 60 °C and 45 s at 72 °C.

Characterization of intron/exon structures. We identified BAC clones corresponding to the human *CDY1*, human *CDY2*, human *CDYL* and mouse *Cdyl* genomic loci by hybridization screening on high-density filters

(Research Genetics) of CITB BAC libraries prepared from human or mouse genomic DNA. We then used these BAC clones to characterize the intron/exon structures of the genes. We designed a series of PCR assays, based on the cDNA sequence of each gene, each yielding a cDNA product of approximately 50 bp and collectively encompassing the entire cDNA sequence. We then repeated each of these PCR assays using the corresponding BAC as template. In some cases, we obtained PCR products of identical length using cDNA or genomic BAC as template, indicating that no introns were located between the two primers. In other cases, a larger PCR product was obtained using the BAC as template (in some cases long-range PCR employing TaqExtender enzyme (Stratagene) was required), indicating the presence of an intron. We sequenced all intron-containing PCR products to identify intron/exon boundaries precisely.

Radiation hybrid mapping of human *CDYL*. Using PCR, we tested DNAs from the 93 hybrid cell lines of the GeneBridge 4 panel[17] (Research Genetics) for the presence of *CDYL*. PCR primers were 5′–CTGAGCAGGAGAACATCACC–3′ and 5′–GCTACGGGTGAGCTTGTTTC–3′. Thermocycling conditions were as listed above. Analysis of the results positioned *CDYL* with respect to the radiation hybrid map constructed at the Whitehead/MIT Center for Genome Research[18] (http://www-genome.wi.mit.edu/cgi-bin/contig/phys_map).

Genetic mapping of mouse *Cdyl*. We typed genomic DNA from the BSS ([C57BL/6JEi×SPRET/Ei]F1 female×SPRET/Ei male) backcross panel (Jackson Laboratory) for a *Cdyl* polymorphism by PCR. PCR primers were 5′–AAAAATGACCCTCACTAAGTTAC–3′ and 5′–AGGCTCCCTGCAGTAAGTA–3′. Thermocycling conditions were as listed above. This PCR assay yielded a 53-bp product when C57BL/6 genomic DNA was used as template, but no product with *Mus spretus* DNA (because of sequence polymorphism at priming sites). Analysis of the results positioned *Cdyl* with respect to the genetic linkage map maintained at the Jackson Laboratory (http://www.informatics.jax.org/map.html).

Southern- and northern-blot analyses. Each Southern-blot lane contained genomic DNA (7 µg). We incubated blots overnight at 65 °C in Na_iPO_4 (0.5 M), 7% SDS, followed by three washes of 15 min each at 60 °C in 1×SSC, 0.1% SDS. For northern-blot analysis, commercial filters (Clontech) doped with poly(A)+ RNA (2 µg/lane) were incubated under the same conditions, then washed at 65 °C in 0.1×SSC, 0.1% SDS.

GenBank accession numbers. Human *CDY1* major transcript, AF080597; human *CDY1* minor transcript, AF000981 (ref. 1); human *CDY2*, AF080598; human *CDYL*, AF081258 and AF081259; mouse *Cdyl*, AF081260 and AF081261.

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