Identification and characterization of the cynomolagus monkey chromodomain gene cynCDY, an orthologue of the human CDY gene family*

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Microdeletions within the AZF (azoospermia factor) a, b and c regions of the Y chromosome can be detected worldwide in 1–10% of infertile men. AZFc, containing genes such as DAZ, CDY, RBMY and others, is most frequently deleted and associated with oligo- or azoospermia. The function of the different genes within AZFc is not yet understood. Here we report the identification and first characterization of the cynomolagus monkey (Macaca fascicularis) homologue of the human CDY gene. cynCDY encodes a 541 aa protein, which like human CDY possesses two putative functional domains: an N-terminal chromodomain, possibly involved in heterochromatin interactions, and a C-terminal domain showing similarity to enoyl-CoA-isomerase, which is involved in fatty acid oxidation. Northern analysis and in-situ hybridization experiments revealed testis- and stage-specific expression of cynCDY mRNA, mainly confined to round and elongating spermatids. Fluorescence in-situ hybridization (FISH) performed on monkey metaphase chromosomes displayed exclusively Y-specific signals in Yq12.1. Using fibre FISH, short signal stretches that indicate the presence of three CDY copies could be visualized, although their integrity or function remains unknown. cynCDY is similar to human CDY with features of a retrotransposon, but different in the 3’ UTR. It seems to represent a more ancestral form of CDY and its characterization yields insights into the evolution of candidate genes for AZF.

Key words: AZF/azoospermia/CDY/microdeletions/Y chromosome

Introduction

Microdeletions of the Y chromosome have been described in 1–10% of patients with idiopathic male infertility and are associated with severely reduced sperm concentrations <5×10⁶ sperm/ml (Hargreave, 2000; Simoni, 2001). Three distinct AZF (azoospermia factor) regions (AZFa,b,c) required for normal spermatogenesis have been defined on the Y chromosome (Vogt, 1998). AZFc is the most frequently deleted region, comprising 80% of all detected microdeletions (Kostova and Gromoll, 2001; Maurer et al., 2001). AZFc deletions manifest in an azoospermic or severely oligozoospermic phenotype. In most cases histological evaluation of testicular biopsies reveals a complete Sertoli cell-only (SCO) syndrome characterized by the absence of germ cells or, in the case of oligozoospermia, by a patchy appearance of the testis with SCO tubules adjacent to tubules with some spermatogenic activity (Krausz et al., 1999).

The drastic effects of an AZFc microdeletion on spermatogenesis imply that genes essential for spermatogenesis are located in this region of the Y chromosome. The most common type of AZFc deletion is large (3.5 Mb) and results in the complete loss of several testis-specific genes or gene families (Saut et al., 2000; Kuroda-Kawaguchi et al., 2001). The DAZ, RBMY, BPY2, PRY and CDY1 genes can be allocated within AZFc and each of them may be a candidate gene for AZF (Lahn and Page, 1997; Hargreave, 2000; Tifford et al., 2001).

The gene content of AZFc has evolved by distinct molecular processes. In the case of the DAZ gene family, the entire genomic segment containing the autosomal DAZ homologue was duplicated and transposed to the Y chromosome, whereas in the case of CDY, a fully processed reverse-transcribed cDNA was integrated into a transcriptionally permissive locus on the Y chromosome. Amplification of both genes gave rise to the DAZ and CDY gene families and thus, transposition (DAZ), retroposition (CDY) and subsequent amplification represent mechanisms for gene building during evolution of the human Y chromosome (Glaser et al., 1998a; Lahn and Page, 1999; Lahn et al., 2001). In the human, CDY evolved from the Y chromosomal retrotransposition of a transcript generated from the autosomal single copy CDYL (CDY-like) gene. Thereafter, CDY underwent gene duplications on the Y chromosome, giving rise to at least three CDY copies, two of them designated CDY1 (with a major and a minor splice variant), located in interval 6F on the long arm of the Y chromosome and one, designated CDY2, located in interval 5L. The CDY1 major transcript and CDY2 completely lack any intronic sequences, as one would expect from retrotransposons, while the CDY1 minor transcript has regained some intronic sequences, giving rise to a CDY gene reminiscent of exon–intron structure. The CDY genes are expressed only in the testis, whereas the CDYL gene is ubiquitously expressed (Lahn and Page, 1999; Saxena et al., 2000).

*Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no.: AJ314841).
The process of gene building on the Y chromosome occurred within the last 100 million years, making the DAZ and CDY gene families young genes in evolutionary terms. CDY and DAZ genes can be detected on Y chromosomes of New World monkeys (only CDY, not DAZ), Old World monkeys, great apes and humans, but are not present on the Y chromosome of non-primate species (Reijo et al., 1995; Gromoll et al., 1999; Lahn and Page, 1999). Thus, classical animal models for the study of spermatogenesis, like rats and mice, completely lack the DAZ and CDY gene family. Therefore, functional studies of the expression of these genes or knock-out experiments are either not possible or can only be approached by knock-in strategies (Slee et al., 1999). The divergent gene content on the Y chromosome of different species is scientifically challenging and requires more suitable animal models. The non-human primate, the cynomolgus monkey Macaca fascicularis, provides an animal model which is more closely related to the human than rats or mice with regard to genetic content and hormonal regulation of spermatogenesis.

In our attempt to characterize the genes from the AZFc region of the human in the monkey model, we have recently isolated and characterized the monkey homologue of the human DAZ gene family. In-situ hybridization was performed on 1% agarose gel containing formaldehyde. The gel was blotted at 37°C for 2 h in 1× SSC for 2×15 min, in 1× SSC for 2×15 min, in buffer containing 20 µg/ml RNase A for 30 min, and in 0.1× SSC for 2×30 min. Hybridization signals were detected by incubation with an anti-DIG-AP antibody and subsequent staining with nitroblue tetrazolium chloride (NBT) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate solution (Boehringer). Sections were counterstained with haematoxylin. After conventional mounting, tissue sections were examined by light microscopy. Images were taken with CCD AxioCam (Carl Zeiss Jena GmbH, Köl n, Germany). Staining of the seminiferous tubules was performed corresponding to the 12-stage classification proposed by Clermont and Antar (Clermont and Antar, 1973).

Materials and methods

cDNA library screening

The monkey testis cDNA library was constructed using the oligo dT primed cDNA synthesis kit (Stratagene, Heidelberg, Germany) employing the Uni-ZAPXR unidirectional phagemid vector, in collaboration with the Institute of Hormone Research, Hamburg, Germany. Plating of recombinant phages, generation of replica filters and hybridization were performed according to standard protocols. Hybridization was carried out using the full length human CDY cDNA as a probe at 60°C for 16–18 h. Filters were washed at a final stringency of 1× standard saline citrate (SSC)/0.1% sodium dodecyl sulphate (SDS) at 60°C and exposed for 1–2 days at ~80°C using intensifying screens. Positive clones were purified by plaque isolation and two additional screening procedures. Phagemids were excised according to the manufacturer’s protocol and the corresponding cDNAs sequenced from both directions.

Northern blot hybridization

Total RNA from human and cynomolgus monkey testis tissue was isolated by the Ultraspec method (Biotec, Houston, USA). RNA was precipitated with isopropanol at 4°C overnight, washed with 75% ethanol, dissolved in DEPC-treated water and the RNA concentration was determined photometrically. Electrophoresis of the samples, each lane containing 20 µg RNA, was performed on a 1% agarose gel containing formaldehyde. The gel was blotted onto a nylon membrane (Amersham, Braun schweig, Germany) and fixed by cross-linking through UV irradiation. Filters were prehybridized at 68°C for 1 h in ExpressHyb (Clontech, Heidelberg, Germany) containing 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed for 2 h under the same conditions with addition of the [32P] dCTP labelled cDNA probe corresponding to the entire coding sequence of cynCDY (1.6 kb), to a final concentration of 1×106 cpm/ml. In addition, a β-actin cDNA probe was used as loading control. The HighPrime kit (Boehringer, Mannheim, Germany) was used for labelling the probes. After hybridization, the filters were washed 4× for 10 min with continuous agitation in 2× SSC, 0.1% SDS at room temperature and 2× for 15 min in 0.1× SSC, 0.1% SDS at 60°C. Blots were exposed overnight to phosphor-screen imaging plates at 4°C and scanned using the Storm 860 phosphorimag er (Molecular Dynamics, USA).

In-situ hybridization

Java monkey testis tissue was bouin-fixed and embedded in parafilm using routine procedures. Sections of 5 µm thickness were mounted on Super Frost Plus slides (Langenbrinck, Emmendingen, Germany). After deparaffinization and rehydration, sections were treated according to a previously described procedure (Gromoll et al., 1997) and prehybridized for 2 h at 37°C in 50 µl of hybridization buffer (50% formamid, 2× SSC, 1× Denhardt’s, 250 µg/ml yeast tRNA and 125 µg/ml salmon sperm DNA) in a humid chamber. In-situ hybridizations were performed for 18 h at 37°C in 40 µl of hybridization buffer containing 10% dextran sulphate and 70–100 ng DIG-labelled sense or antisense cRNA probe corresponding to the entire open reading frame (ORF) sequence of cynCDY. Probes were generated by an Sp6 or T7 RNA polymerase in-vitro transcription using a DIG-RNA labelling kit (Roche Diagnostics, France). After hybridization, sections were washed at 37°C in 2× SSC for 2×15 min, in 1× SSC for 2×15 min, in buffer containing 20 µg/ml RNase A for 30 min, and in 0.1× SSC for 2×30 min. Hybridization signals were detected by incubation with an anti-DIG-AP antibody and subsequent staining with nitroblue tetrazolium chloride (NBT) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate solution (Boehringer). Sections were counterstained with haematoxylin. After conventional mounting, tissue sections were examined by light microscopy. Images were taken with CCD AxioCam (Carl Zeiss Jena GmbH, Köl n, Germany). Staining of the seminiferous tubules was performed corresponding to the 12-stage classification proposed by Clermont and Antar (Clermont and Antar, 1973).

Southern blot hybridization

A total of 20 µg EcoRI digested DNA from female and male adult humans and monkeys was agarose gel electrophorized and blotted onto nylon membrane (Amersham). The hybridization conditions were identical to the Northern blot procedure, except for the hybridization and washing temperatures which were lower (60 and 50°C respectively). Exposure and scanning were as previously described.

PCR amplification

Genomic DNA was obtained from macaque and human peripheral leukocytes using the Nucleon Kit II (Scotlab, Wiesloch, Germany). PCR amplification of genomic DNA from cynCDY and human CDY1 minor form was performed using the oligonucleotide primers J130, CCGTTCTTCTCTGACGTTCTC (forward for both species); macaqueCDY-2r, CTTTACCATGGATTCGACCC (reverse for macaque); CDYa-exon2-2r, CATTCCGTGTCTTCCTGCGGAGG (reverse for human and specific for CDY1 minor). The following products were obtained: a 1610 bp fragment from macaque DNA and a 2076 bp fragment from human DNA. PCR conditions were: 20 s at 94°C for initial denaturation, then 35 cycles of 20 s at 94°C, 30 s at 58°C, 2 min 30 s at 72°C, and then a final extension of 4 min at 72°C. The PCR products were run on a 1% agarose gel.

Fluorescence in-situ hybridization (FISH)

Chromosome preparation

Chromosome spreads for fluorescence in-situ hybridization (FISH) were prepared from PHA-stimulated peripheral blood lymphocytes taken from male cynomolgus monkeys (M. fascicularis) as previously described (Schempp et al., 1995). Slide preparations were checked for well-spreading and plasma-free preparation of metaphase plates. Only slides from such batches judged as optimal metaphase preparations were considered for FISH, and were stored at ~80°C until use.

Released chromatin preparation

Extended Y chromatin structures from interphase nuclei were generated according to previously published protocols (Fidlerova et al., 1994).

FISH

Prior to FISH, the slides were treated with RNase followed by pepsin digestion as previously described (Ried et al., 1992). FISH using genomic lambda clones for cynDAZ, corresponding to genomic region of exon 2 to exon 6 of the monkey cynDAZ gene, and the full length cynCDY cDNA on chromosome spreads of the cynomolgus monkey (M. fascicularis) essentially followed the methods as described (Schempp et al., 1995). FISH of the full length cynCDY cDNA to released chromatin preparations was performed accordingly.

Fluorescence microscopy and imaging

Preparations were evaluated using a Zeiss Axioshot epifluorescence microscope equipped with single-bandpass filters for excitation of red, green and blue (Chroma Technologies). During exposures, only excitation filters were changed, allowing for pixelshift-free image recording. Images with high

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and
Results

The cyn (cynomolgus) CDY cDNA and the putative amino acid sequence derived

Screening of a monkey testis cDNA library using human CDY cDNA as a probe yielded several positive clones. Further analysis of these clones by DNA sequencing revealed that three out of four clones contained partial sequences with different 5’ extensions, and that one clone contained a full length cDNA with 1879 nucleotides. Comparison of all four clones revealed complete sequence identity and high similarity to human CDY. The lack of clones displaying other CDY transcripts indicates that the cDNA identified is the predominantly expressed cynCDY isoform.

Inspection of the cynCDY cDNA (accession no. AJ314841) revealed that the ORF consists of 1627 nucleotides and is preceded by a poly A tail. No classical polyadenylation signal could be identified. The ORF contains a full length cDNA with 1879 nucleotides. Comparison of all four clones revealed complete sequence identity and high similarity to human CDY. The lack of clones displaying other CDY transcripts indicates that the cDNA identified is the predominantly expressed cynCDY isoform.

The putative amino acid sequence derived from the cynCDY cDNA displays a protein with 541 aa and a molecular mass of 60 kDa. The pI is 9.06 and there are no specific hydrophobic or hydrophilic domains within the protein detectable using the DNAsis software program.

cynCDY protein displays features of chromo- and catalytic domain

A protein domain search within the cynCDY amino acid sequence showed, as with human CDY, the presence of two putative domains. In the N-terminal region (covering aa 1 to 61), cynCDY displays similarities (from 69 to 38%) to proteins having a chromodomains which can be involved in interactions with heterochromatin sites (Ball et al., 1997; Figure 1). The homology is conserved over a range of different species and organisms. A second domain with similarity varying from 70 to 35% can be allocated to the C-terminal part of cynCDY (aa 246–541). It displays motifs of a catalytic domain, with features corresponding to the enoyl-CoA isomerase enzyme (Jannsen et al., 1994). Both domains are connected by an intermediate region with no apparent identity.

Detailed amino acid sequence comparisons of cynCDY to the three human CDY isoforms and the autosomal homologue CDYL revealed closest identity to CDY2 with respect to amino acid number (541 aa in both genes) and amino acid sequence similarity (82%, Figure 2). However, this is not significantly different from the CDY1 major and minor proteins. When cynCDY is compared with CDYL, overall identity drops to 58%. Amino acid similarity between cynCDY and the different CDY genes reaches 97% within the chromodomain and drops to 87% within the catalytic domain. Identity between cynCDY and CDYL within both domains is not changed (70%; data not shown).

cynCDY is present on the Y chromosome of the cynomolgus monkey as a retrotransposon

To investigate whether cynCDY is present as a retrotransposon on the Y chromosome, similar to CDY in the human, primers located 5’ and 3’ of the cynCDY cDNA were used for PCR amplification using female and male monkey DNA. An amplicon could only be generated from genomic male monkey DNA and not from female monkey DNA, indicating the Y chromosomal localization (Figure 3). The size of the amplicon obtained corresponds exactly to the identified cDNA of cynCDY, indicating that no additional intronic sequences are present. The human CDY1 minor form was amplified, again using specific primers located 5’ and 3’ within the CDY cDNA. Instead of the calculated 1.6 kb based on the cDNA of CDY1, an amplicon of ~2.1 kb could be detected. This difference in the size of cDNA and genomic DNA is caused by the additional intron present in the human CDY1 (Lahn and Page, 1999). Thus the amplicon displays the whole exon–intronic structure of CDY1, a genomic organization not present in the monkey.

Expression analysis of cynCDY mRNA

Tissue-specific expression, transcript size and level of expression was investigated by hybridizing total RNA from cynomolgus (macaque) monkey, marmoset and human using a radioactively labelled 1.6 kb cynCDY probe encompassing the whole ORF of cynCDY. A hybridization signal could only be detected in testicular tissues, but not in mouse testis and not in other monkey organs such as male liver and kidney or female uterus (Figure 4). For cynCDY only one transcript of ~2.4 kb, as calculated from the migration pattern of the corresponding 28S and 18S band, was visible. Densitometric analysis of the CDY signals obtained after normalization with β-actin revealed a 3-fold and 5-fold higher expression in the cynomolgus monkey compared with the marmoset and human respectively. However, it is possible that the different expression levels could in part be affected by different hybridization kinetics when using a cynCDY probe (88% identical to human CDY). By in-situ hybridization on testis sections, cynCDY mRNA could be localized in round and early elongating spermatids (Figure 5A,B). Light microscopy investigation showed that round spermatids express cynCDY mRNA during stages I–VII with a maximum in stage VII. Clear hybridization signals in elongating spermatids were confined to stages VII–IX, and were faint in stages X–XII and I–V. Thus, in quantitative terms, expression was maximal during stages VII–IX. Based on our in-situ hybridization experiments we cannot completely exclude cynCDY mRNA expression in other germ cell types; however, the level of expression must be low. No signals were detected in testis sections hybridized with a sense probe (Figure 5C). A schematic drawing depicting the relative cynCDY mRNA distribution during monkey spermatogenesis is shown in Figure 5D.

Southern blot hybridization of cynCDY

Southern blot hybridization of female and male EcoRI digested genomic DNA from human and monkey was performed with 1.6 kb cynCDY probe corresponding to the whole cynCDY coding sequence. Several bands were visible in female and male humans and monkeys (Figure 6). Additional specific signals could be obtained only in males, indicating the Y chromosomal location of CDY. The presence of more than one specific band in the monkey and human males may be caused by the presence of several CDY genes. In the marmoset male monkey, only a few additional bands compared with the female were observed. This might indicate the presence of only a single Y chromosomal gene in this species. Signals present in both sexes might confer cross-hybridization on either the autosomal CDYL genes and/or the chromo- or catalytic domains from other genes.

FISH and fibre-FISH analysis

The Y chromosomal location of DAZ and CDY genes in the cynomolgus monkey (M. fascicularis) was defined by applying multicolour FISH of cynomolgus-specific gene probes to metaphase chromosome spreads of this monkey. DAZ and CDY sequences were exclusively mapped in the proximal long arm region of the tiny acrocentric Y chromosome of the cynomolgus monkey (Figure 7) (Glaser et al., 1998b).
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Figure 1. Alignment of cynCDY amino acid sequence using the BLAST programme. The chromodomain and catalytic domain are indicated by black bars. Identical amino acids are marked and numbering indicates the positions of the aligned sequences; the number after the slashes indicates the whole amino acid number of the corresponding protein. The following proteins were found to match with either the chromo or the catalytic domain of cynCDY (the accession numbers from the SwissProt database are given in brackets): human CDYL (AAD22734.1); mouse brain protein (BAA95081.2); Cl.fulvum Cft-1 LTR-retrotransposon (AAF21678.1); mouse CBX3 (NP_031650.1); Drosophila HP1 (P05205); human CBX3 (NP_009207.1); G.gallus CHCB2 (BBA33401.1); F.yeast CLR4 (O60016); human D3 D2-enoyl-CoA isomerase (O75521); mouse D3, D2-enoyl-CoA isomerase (NP_035998.1); C.carpio ACBP/ECHM (AAC19408).

To determine the number of CDY genes on the cynomolgus monkey Y chromosome, we applied high-resolution FISH with a cynomolgus-specific CDY gene probe to extended interphase Y chromatin (FISH). As shown in Figure 8, three short signal stretches, numbered from 1 to 3, can be distinguished on the Y chromosome. The four different Y chromatin fibres show typical variations of signal distribution after fibre FISH with the full length cynCDY cDNA as a probe. The slight differences observed are due to the applied technique of chromatin release.

If one supposes a maximum release of DNA (1bp = 3.4 Å), the bar of 10 μm in Figure 8 would comprise some 30 kb. This gives a length of the whole CDY cluster of ~70 kb. However, it should be noted that the ‘fibre FISH’ technique applied here does not allow for an accurate length calibration of chromatin fibre. Thus an exact measurement in terms of fibre length per bp of DNA is not possible.

Discussion

CDY belongs to a group of gene families which, like DAZ, RBMY and BPY, are putative candidate genes for AZFc. Unlike the RBMY genes, DAZ and CDY are phylogenetically young genes on the Y chromosome, and are present only in primates and humans. Only indirect evidence is currently available concerning their role in spermatogenesis. In most cases, the deletion of each of the genes within AZFc leads to severe spermatogenic failure, resulting in oligozoospermia.

Evolutionary aspects of cynCDY

CDY evolved from an initial retrotransposition of the fully processed transcript of the autosomal CDYL gene into an ancient form of the Y chromosome and is present in simian primates, including apes, Old World and New World monkeys. Thus the retrotransposition event must have occurred ~40–50 million years ago in the simian lineage after its divergence from prosimians, but before the split between Old and New World monkeys (Sassaman et al., 1997; Lahn and Page, 1999). CDY persisted on the Y chromosome throughout evolution and underwent further changes and amplifications.

FISH experiments on monkey chromosomes revealed only one positive signal for cynCDY within the proximal long arm of the Y chromosome. To determine the accurate gene copy number, fibre FISH was performed and three distinct signals, corresponding to three gene copies, on the cynomolgus Y chromosome could be distinguished. However, there is no evidence that all of them are functional. Screening of the monkey testis cDNA library gave no hints that the different copies can correspond to different gene forms, which might indicate the presence of only one type or only one functional copy of CDY on the monkey Y chromosome.

Homology plots revealed that cynCDY shows closest similarity to CDY2, but this is not significantly different from CDY1. Thus, it is difficult to estimate the features of the ancient form which was first retrotransposed to the Y chromosome. Inspection of the 3’UTR of human CDY and cynCDY genes provides interesting details. In CDY2 and CDY1 major transcripts polyadenylation occurs close to or directly after the stop codon and polyadenylation signals can be identified within the ORF, e.g. the polyadenylation signal AATAAA in CDY2 is located just 15 nucleotides before the translational stop codon. Such signals cannot be observed in the corresponding autosomal CDYL gene, thus these sites must have arisen after retrotransposition through mutations that generated appropriate signal
Figure 2. Amino acid sequence comparison of macaque cynCDY and human CDY genes (CDYL, CDY2, CDY1 minor and CDY1 major splice forms). Alignment was performed using DNasis software (Hitachi, Japan). The chromodomain and the catalytic domain are indicated by grey boxes; amino acids are numbered and non-matching amino acids in all sequences are highlighted in black; arrows below the sequence indicate identical amino acids between cynCDY and CDYL, but different from CDY1 and CDY2; asterisks indicate identical amino acids between cynCDY and CDY2, but different from the CDY1 amino acid sequences, and dots indicate identical amino acids between cynCDY and CDY1 isoforms, but different from CDY2 and CDYL.

sequences (Lahn and Page, 1999). Nucleotide sequence comparison of cynCDY with CDY2 revealed that the internal polyadenylation motif present in the human is not present in the monkey (AATCAA instead of AATAAA). This might be the reason that cynCDY is polyadenylated further downstream, although classical sites for polyadenylation could not be identified. Furthermore, cynCDY still possesses a relatively large 3’UTR with 229 nucleotides. Comparison of the 3’UTR in the human CDY and the monkey cynCDY revealed high identity with 62% when compared with 35% similarity between CDYL and CDY1 minor transcript. The different polyadenylation
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Figure 5. Micrographs depicting the expression of cynCDY mRNA as revealed by in-situ hybridization in cynomolgus monkey testis. The stages of the cycle of seminiferous tubules are marked by Roman numerals. (a) Testis tissue section hybridized with a cynCDY antisense cRNA probe corresponding to the entire coding sequence; specific signals are detectable in round and elongating spermatids in stage VIII; a dashed line marks the region shown in 5b; (b) higher magnification of (a): cytoplasmic cynCDY expression in spermatids—arrows indicate positively stained round or early elongating spermatids; (c) corresponding to (a): no staining could be seen in the control section hybridized with a sense cRNA probe; (d) schematic representation of cynCDY mRNA expression in the different germ cell types; a grey to black scale represents the signal distribution evaluated by light microscopy. (a,c) Scale bar, 50 µm; (b) scale bar, 20 µm.

patterns between human and monkey CDY favours a model in which the ancient form of the retrotransposed CDY had a 3' UTR containing a polyadenylation site which, with the introduction of mutations during evolution, led to the CDY transcripts currently seen.

The chromo- and catalytic domains of the CDY genes

Similarities between human and monkey CDY in the chromo and catalytic domains are surprisingly high when compared with 82% overall amino acid identity. In the case of the chromodomain this value reaches 97%. This implies that the domains of the CDY genes lie under some evolutionary constraints, indicative of a functioning protein.

Proteins containing a chromodomain such as cynCDY are generally believed to be involved in an interaction with heterochromatin sites to mediate gene silencing (Ball et al., 1997; Bannister et al., 2001). Concerning the catalytic domain, sequence identities are not as evident as they are for the chromodomain. The catalytic domain of cynCDY shows a pattern homologous to a key enzyme in mitochondrial β-oxidation of unsaturated fatty acids, enoyl-CoA isomerase. By amino acid comparisons of the autosomal mouse and human CDYL genes it becomes clear that the catalytic domain is highly conserved (99%), indicating a crucial function of this domain.

cynCDY mRNA expression

Northern blot experiments revealed testis-specific cynCDY mRNA expression and transcription levels significantly higher than in the human testis. Even the expression of CDY mRNA in another primate species, the marmoset monkey (Callithrix jacchus), seems to be higher than in the human. These differences might be due to some kind of gene silencing in the human, possibly induced by additional mutations in putative regulatory regions of the promoter or by new mutations, which could effect turnover of the human CDY mRNA. However, it is also possible that the observed differences in the expression levels are in part affected by different hybridization kinetics, given that we used a cynCDY probe. Further studies on the regulation and onset of expression should clarify the differences observed in CDY expression.

Maximum cynCDY mRNA expression was detectable by in-situ hybridization during the advanced stages of spermatogenesis in round and elongating spermatids. This expression pattern may not necessarily
Figure 6. Southern blot of male and female EcoRI digested genomic DNA from human, macaque and marmoset hybridized with a 1.6 kb cDNA fragment encompassing the entire open reading frame of cynCDY sequence. In all males, several bands are visible, corresponding to the Y-encoded CDY genes. Marker DNA fragments are indicated on the left side. f = female; m = male.

Figure 7. Two colour FISH of a full length cyn CDY cDNA (green) and cynDAZ probe (red) encoding exon 2–6 on metaphase spreads of *M. fascicularis*. Note that the CDY and DAZ probes hybridize to the long arm of the Y chromosome (higher magnification is shown in the lower right corner). The Y centromere is marked by a small bar. A schematic drawing of the macaque Y chromosome indicating the CDY and DAZ location within Yq12.1 locus is shown in the lower left corner.

Figure 8. High-resolution FISH of a full length cynCDY cDNA on four examples of extended Y chromatin in interphase of *M. fascicularis*. Three CDY signal stretches numbered from 1 to 3 may be interpreted as three CDY copies on the four different Y chromosomes.

Conclusions
In summary, the isolation and characterization of the monkey cynCDY homologue of the human CDY gene family yields insights into the evolution of Y chromosomal genes. It demonstrates that three CDY gene copies, without proof of function, are present in the cynomolgus monkey, similar to the human where at least three copies have been distinguished so far. Further rearrangement events of the CDY genes have probably occurred only in the human. This is in agreement with the recently cloned cynomolgus monkey DAZ gene where a more ancient form could be found. Delineation of the expression patterns during spermatogenesis and characterization of conserved domains within the CDYs of human and monkey should now enable functional studies, e.g. interaction of CDY with heterochromatic sites, to prove that this gene is functional and might be a candidate gene for AZF.
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