

The Old World monkey *DAZ* (Deleted in AZoospermia) gene yields insights into the evolution of the *DAZ* gene cluster on the human Y chromosome

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The *DAZ* gene cluster on the human Y chromosome is a candidate for the Azoospermia Factor (AZFc). According to the current evolutionary model, the *DAZ* cluster derived from the autosomal homolog *DAZL1* through duplications and rearrangements and is confined to Old World monkeys, apes and humans. To study functional and evolutionary aspects of this gene family we have isolated from a cynomolgus (Old World) monkey testis cDNA library the Y chromosomal *cynDAZ* and the autosomal *cynDAZL1* cDNA. *cynDAZL1* contains one *DAZ* repeat and displays high homology to human *DAZL1*. *cynDAZ* comprises 11 repeats, each consisting of exons 7 and 8, whereas the human *DAZ* cDNA repeat units contain predominantly exon 7. Genomic studies revealed the same amplification events of a 2.4 kb genomic unit encompassing exons 7 and 8 in both species, indicating that after splitting of the two lineages, in the human mainly exon 8 was converted to a pseudoexon by splice site mutations. The structural features of *cynDAZ* reveal a more detailed model for the sequence of events leading to the present form of human *DAZ*. Thus, in a monkey species *DAZ* is present in a form more ancestral than that of the human. Studies on the immunolocalization of *cynDAZ/DAZL1* in cynomolgus monkey testis revealed a biphasic expression pattern with proteins being detectable in A-pale to B-spermatogonia, late spermatocytes and spermatids, but not in early spermatocytes and late spermatids. In contrast, in the marmoset monkey, an animal lacking *DAZ*, *DAZL1* protein was only expressed in late spermatocytes and early spermatids. These findings point to an additional function of *cynDAZ/cynDAZL1* during spermatogenesis in the Old World monkey not needed in the New World monkey.

INTRODUCTION

The existence of an Azoospermia Factor located in the distal part of the Y chromosome, bearing genes crucial for spermatogenesis, was first postulated by Tiepolo and Zuffardi (1) and was further supported by the identification of microdeletions in the Yq11 region of oligo- and azoospermic men (2). Multicenter studies of infertile men (3) led to the identification of three different AZF loci (AZFa, -b and -c). Microdeletions of AZFa–c could be detected in 5–15% of the infertile men, suggesting that the deleted regions of the Y chromosome contain genes essential for spermatogenesis (4,5).

One candidate gene in the AZFc region is the recently described *DAZ* gene (Deleted in AZoospermia) which can be mapped to Yq11.23 (2). It encodes a putative RNA-binding protein with one RNA-binding domain and a characteristic 24 amino acid *DAZ* repeat unit. *DAZ* was acquired by the Y chromosome from an autosomal homolog *DAZL1* located on chromosome 3p24 (6–9). *DAZ* shares high homology with *DAZL1*, but unlike *DAZL1*, which is a single copy gene, it has undergone a complex series of duplications and rearrangements resulting in a polymorphic gene family (10). According to the current hypothesis for the evolution of *DAZ*: (i) a complete copy of autosomal *DAZL1* was transposed to the Y chromosome; (ii) within the newly transposed gene a 2.4 kb genomic segment encompassing exons 7 and 8 was tandemly repeated and in most of the amplified units exon 8 degenerated, resulting in mainly exon 7 repeat units transcribed as the functional derivative of these amplifications; and (iii) the whole transcription unit was amplified, giving rise to a multicopy gene family (6,10).

The acquisition of *DAZL1* by the Y chromosome must have been a recent event in evolution, since Southern blot analysis (2,7,9) and PCR experiments (11,12) demonstrated that *DAZ* is present on the Y chromosome of Catarrhini (Old World monkeys, apes and humans), but not in Platyrrhini (New World monkeys) and other mammals. Since the two monkey lineages split ~40 million years ago (13), the translocation of *DAZ* to the Y chromosome must have taken place thereafter.

DAZL1 or its homologs is expressed exclusively in the germ cells of female and male gonads of various species (11,14–16), whereas *DAZ* is most highly expressed in human spermatogonia

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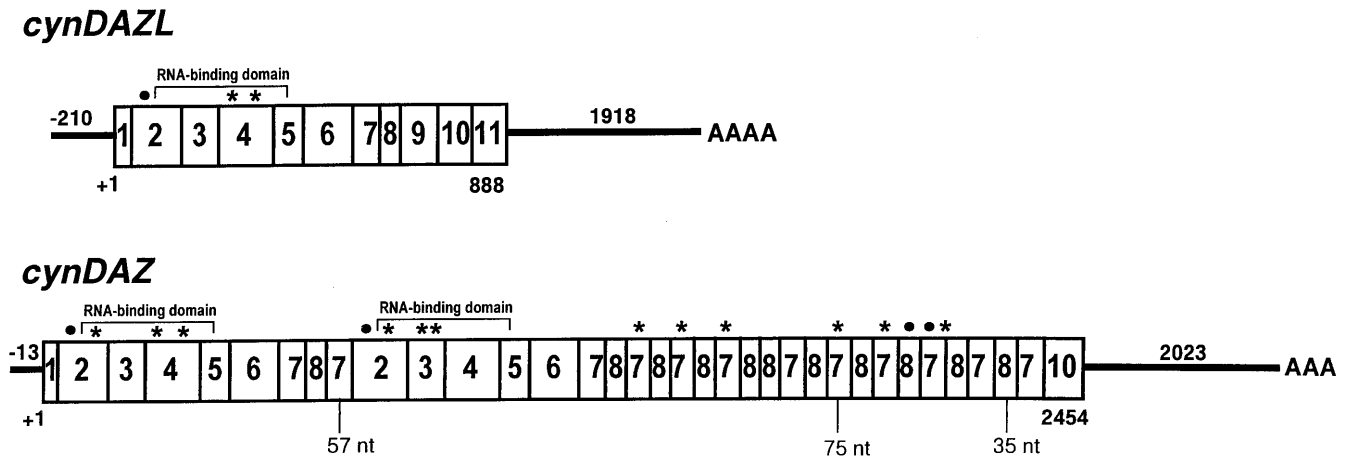


Figure 1. Schematic graphic representation of *cynDAZL1* and *cynDAZ*. The boxes indicate the ORF starting with +1. Numbering of the different exons and their corresponding sizes was done in homology with human *DAZL1* and *DAZ* (6). Untranslated regions are shown by a line and the location of the RNA-binding domain by brackets. The putative glycosylation sites are marked by circles and the putative phosphorylation sites by asterisks. Within the UTR of *cynDAZL1* and *cynDAZ* one polyadenylation signal (AATAAA) for each cDNA can be identified. The sequences have been deposited in the EMBL database under accession nos X99971 for *cynDAZL1* and AJ012216 for *cynDAZ*.

(17). In mice expression of the autosomal *DAZL1* gene is confined to B-spermatogonia, early spermatocytes, and highest expression can be observed in pachytene spermatocytes (18). Elimination of the *DAZL1* gene in knock-out mice resulted in the complete absence of germ cells beyond the spermatogonial stage, indicating a crucial function of *DAZL1* before or during the onset of meiotic cell divisions (18). Similarly, in the fruitfly, the loss of *boule*, a *DAZL1* homolog, caused a meiotic arrest of germ cells (19).

Old World monkeys represent important and valuable animal models for the study of many aspects of human reproduction. Among the various species in use, the cynomolgus monkey (*Macaca fascicularis*) is particularly well characterized with regard to testicular physiology and gametogenesis (20–22). The fact that Old World monkeys possess a Y-chromosomal *DAZ*, which is absent in other animal models, renders them an exceptional animal suitable for the functional analysis of *DAZ* and its importance for human spermatogenesis. Accordingly, we have cloned the autosomal and Y-chromosomal *DAZ* homologs of the cynomolgus monkey *M.fascicularis* and characterized their expression.

RESULTS

The autosomal *cynDAZL1* cDNA

On screening the monkey testis cDNA library using a human *DAZL1* cDNA probe, five positive clones were identified. Four of the cDNA clones turned out to be identical on restriction site and sequence analysis. The cDNA consists of 3016 nucleotides encoding 295 amino acids (Fig. 1). The open reading frame (ORF) matches the partial sequence of *cynDAZL1A* cDNA obtained by RT-PCR and was previously published by us (11). Therefore, the full-length cDNA sequence isolated here, including the 3'- and 5'-untranslated region (UTR), was designated as the autosomal cynomolgus *cynDAZL1* cDNA. The deduced protein consists of 295 amino acids with an estimated molecular mass of 33.1 kDa. In the N-terminal part one RNA-binding domain bearing the RNP-2 (VFVGGI) and RNP-1 (KGYGFVSF) motifs

can be allocated. The ORF is preceded by a 210 nucleotide 5'-UTR, and after the stop codon is followed by a 3'-UTR with 1918 nucleotides.

The Y-chromosomal *cynDAZ* cDNA

The *cynDAZ* cDNA sequence (Fig. 1) is a composite of the partial cDNA of the remaining fifth clone and a 5'-RACE product using adult monkey testis mRNA as a template. The 5'-RACE products overlapped the cDNA insert by 50 nucleotides and extended 471 nucleotides further to the 5' end.

The *cynDAZ* cDNA shares high homology with the *cynDAZL1* cDNA, but is much larger and has a more complex structure. It covers 4490 nucleotides with 13 nucleotides for the 5'-UTR and 2032 nucleotides forming the 3'-UTR. Inspection of the ORF, extending from +1 to 2454, revealed the presence of a structure consisting of exons 1–6 (since no data on the genomic organization of the *cynDAZ* gene are available, exon numbering followed the human *DAZ* and *DAZL1* gene structure) followed by exon 7, with the 72 bp repeat unit, and exon 8. However, after exon 8 a truncated 57 nucleotide copy of exon 7 is present, directly followed by another exon 2. The cDNA continues with a stretch of nucleotides corresponding to exons 2–6 and a repeat unit structure of 123 nucleotides consisting of exon 7 (72 nucleotides) and exon 8 (51 nucleotides). In total, 11 such exon 7 and 8 repeat units can be identified. After the fifth repeat, the motif is interrupted by the lack of one exon 7 copy, resulting in two adjacent exons 8. Within the eighth repeat exon 7 possesses 75 instead of 72 nucleotides. In the 11th repeat unit, exon 8 is truncated (35 nucleotides) and the nucleotide sequence proceeds with a single exon 7 followed by exons 10 and 11. Exon 9 is lacking within the cDNA. Experiments in which *cynDAZ* specific primer sequences from exon 11 were used for PCR from male or female monkey genomic DNA gave rise to an amplicon only in the male, suggesting a Y-chromosomal localization of *cynDAZ* cDNA (data not shown).

To exclude the possibility that the *cynDAZ* cDNA structure obtained is due to a failure during the generation of the cDNA library, we performed additional RT-PCR experiments using

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mor.key  CTCTGCCTCTGGCTTTACCAGCAATAGAAAAT-TTGTGAAGAGAAACAG  50
|||||
human   CTCTGCCTCTGGCTTTACCAGCCATAAAAAAATGTTGAAGAGGAAACAG  50

AAATGTTTTGCTATTAATTACTMTTAAATAAAGAACAGGAAGAAGA-AAGG  100
|||||
AAATGTTTTGCTGTTAATTACTGTTAAATAAAAAATAGGAATAAAACAAGA  100

GTATTGCCTCTAAAAACCCCAAGCTGCT-TTCTGCCCCCTCACCATATA  150
|||||
GTATTACGCTAAAAACCCCAAGCTGCTGTT-T-CCCCCT-AT-ATA-A  150

GCAACTTGCTAAAGAGAAT-GAAAGCAGCT--C-T-ATGTATCTGTCTAC  200
|||||
-CTAGTTCCTAAAGC-ATTTGAAAGCAGCTATCGTTATGTCTGTCTAG  200

TTTATTATTCTCTAAGTTTAGCA--TCATCTAGCTATTCTTTATTGCGAG  250
|||||
TATATTCTTCTCTAAGGTTAGCAAAATCATCTAGCTATTCTTTATTGCAA  250

TAATTTCCAGATGCCCTCCTC 262
|||||
TGATTTCCAGATGCCCTCCTC 262

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Figure 2. Nucleotide sequence comparison of the junctions between the different 2.4 kb repeats encompassing exons 7 and 8 of monkey and human. The overall homology is 82% and the junction consensus sequence (CCCCCT) is boxed. The primer sequences used to amplify the 262 bp fragment from genomic DNA from male humans and monkeys are underlined. The human sequences are taken from a *DAZ* cosmid (AC0000021).

monkey testis total RNA. cDNA synthesis using a primer located in the second exon 2 followed by subsequent PCR using primer pairs located in the first exons 2 and 6 (Fig. 1), gave rise to an amplicon identical to the cDNA clone. Similar to this, we also confirmed the presence of the different repeats within the cDNA using primer pairs located in the second RNA-binding domain and exon 11. Both products were sequenced and confirmed the observed cDNA structure of *cynDAZ* (data not shown). However, by using this approach, we cannot exclude the existence of other *cynDAZ* isoforms being expressed in the monkey testis.

The ORF consists of 2454 nucleotides encoding 816 amino acids with a calculated molecular mass of 93.2 kDa and an isoelectric point (pI) of 9.24. Two RNA-binding domains, interrupted by a partial repeat structure, are present in the N-terminal part. The second domain is followed by several repeat units encoded by exons 7 and 8 with 41 amino acids (123 nucleotides) each. Further characteristic features of the putative protein structure include an adjacent exon 8/exon 8 after the fifth repeat unit and a truncated last exon 8 preceding a singular exon 7, which leads to a shift in the ORF terminating translation within exon 10 (Fig. 1)

Comparison of *cynDAZLI* and *cynDAZ* with respect to specific amino acid motifs revealed four putative N-linked glycosylation sites in *cynDAZ* cDNA (Fig. 1) and one in *cynDAZLI* cDNA. The most striking difference is the number of putative PKC phosphorylation sites, where (ST)-x-(RK) is the consensus motif. Two such sites are present in *cynDAZLI*, whereas 12 can be identified in *cynDAZ*, mainly located in exon 7 copies.

Analysis of the genomic repeat structure

In the human, the *DAZ* repeat structure has evolved by tandem amplification of a 2.4 kb genomic segment encompassing exons 7 and 8 (6). The junction sequence between two such repeats consists of a poly(C) sequence, which is present in all human repeats. To investigate whether a similar repeat unit is present on the Y chromosome of the monkey, sequences 5' or 3' of the human repeat unit junction were used for primer selection.

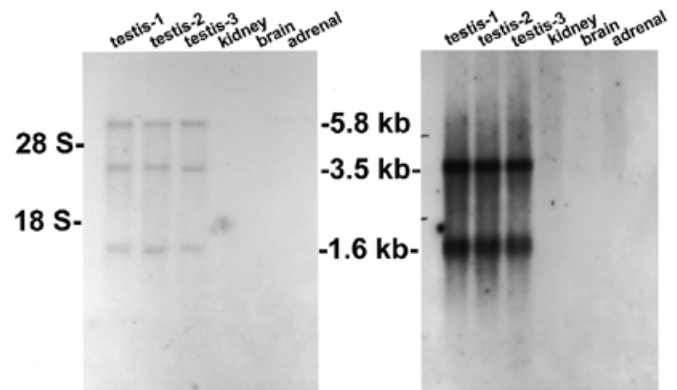


Figure 3. Northern blot hybridization of poly(A)-RNA obtained from different monkey testes (testis-1-3) and from different tissues (kidney, brain, adrenal). The positions of the 18 S and 28 S rRNA bands, which were still visible after mRNA isolation, are indicated. The blot was first hybridized to a specific *cynDAZ* cRNA probe, comprising four exon 7/exon 8 repeats (left side; exposure time 7 days) and, after stripping, reprobed to a cRNA, comprising the whole ORF of *cynDAZLI* (11) (right side; exposure time 2 days).

Following PCR with genomic DNA obtained from male or female monkeys, an amplification product was obtained only in the male monkey. A junction sequence (CCCCCT) similar to that in the human was identified (Fig. 2). The homology of the monkey and human Y region shown in Figure 3 reaches 81%.

Nucleotide and amino acid sequence comparisons

A detailed comparison of the nucleotide and amino acid sequences of monkey *cynDAZ* and *cynDAZLI* and the corresponding human *DAZ/DAZLI* is presented in Table 1. Within the ORF the overall nucleotide sequence homology of the monkey *cynDAZLI* and human *DAZLI* cDNA reaches ~98%. When the 3'-UTRs from the human and monkey autosomal *DAZLI* are compared, a similar homology (94%) is attained.

Similarity drops to 87% (amino acid) and 91% (nucleotide) when autosomal *DAZLI* and Y-chromosomal *DAZ* from the monkey are compared. The predicted overall amino acid sequence is characterized by a high percentage of proline (14%) and tyrosine (9.8%) residues, mainly located in the repeat units. The abundance of these two amino acids are markedly lower in *cynDAZLI* with proline representing 10% and tyrosine 5%, respectively.

Expression of *cynDAZ* and *cynDAZLI*

Northern blot hybridization. Northern blot hybridization of different monkey tissues performed under stringent conditions with a cRNA probe obtained from *cynDAZ* encompassing four exon 7/exon 8 repeats, yielded three testis-specific transcripts, of 1.6, 3.5 and 5.8 kb, respectively (Fig. 3). Reprobing the same northern blot with a cRNA probe covering the ORF of *cynDAZLI* led to the identification of two testis-specific transcripts with 1.6 and 3.5 kb, respectively. Identical transcripts are present in the testes of three different monkeys. No signals could be observed in other monkey tissues.

Table 1. Nucleotide and amino acid sequence comparison of human and monkey *DAZL1* and *DAZ* cDNA

	% Homology					
	<i>cynDAZ/DAZ</i>	<i>cynDAZ/cynDAZL</i>	<i>cynDAZ/DAZL</i>	<i>DAZ/cynDAZL</i>	<i>DAZ/DAZL</i>	<i>DAZL/cynDAZL</i>
Transcribed sequences	90	91.6	91.7	92.6	92.2	98.5
5'-UTR	–	–	–	84.5	85	93.4
3'-UTR	90.5	89.6	88.5	89.6	89.1	94.2
Amino acid sequences	82.6	87.1	87.1	85.1	87.1	98
RNA-binding domain amino acid sequence	85.7	89.3	89.2	89.3	89.3	100

Amino acid sequences were compared up to exon 8, since similarity drops markedly thereafter due to the shift in the ORF by a truncated exon 8 within *cynDAZ* and *DAZ*.

Differential expression pattern of *DAZ/DAZL1* protein in Old World and New World monkeys. In the cynomolgus monkey, belonging to the Old World monkeys, a biphasic testicular expression pattern of *cynDAZ/cynDAZL1* was seen (Figs 4 and 5). The first wave of expression extends from A-pale-type spermatogonia to B-type spermatogonia (Fig. 4a and c) and vanishes in cells just prior to initiation of meiosis (preleptotene spermatocytes). *cynDAZ/cynDAZL1* protein expression could not be detected in leptotene, zygotene and early pachytene stages of the primary spermatocytes (Fig. 4a). The second wave of expression starts with advanced pachytene spermatocytes and is highest in spermatocytes just before germ cell divisions. The protein is still detectable in early spermatid stages (Fig. 4c) but staining diminishes thereafter. Staining was confined to germ cells. Since an antibody directed to a part of the RNA-binding domain common to *cynDAZ* and *cynDAZL1* was used, specific discrimination between the two protein expression patterns is not possible.

In testicular tissue of the marmoset (*Callithrix jacchus*), belonging to the New World monkeys and lacking the Y-chromosomal *DAZ*, specific staining was obtained in advanced primary spermatocytes starting with prophasic stages after the zygotene stage (Figs 4 and 5). Expression was pronounced in late pachytene spermatocytes (Fig. 4d and f) prior to the second meiotic cell division. Expression was also detected in early spermatid stages (Fig. 4d and f). In spermatogonial stages no staining was observed (Fig. 4f). Staining specificity was demonstrated in both species by the lack of signals following omission of the primary antibody (data not shown) or preincubation of the primary antibody with the antigen (Fig. 4b and e).

DISCUSSION

We have cloned the autosomal *cynDAZL1* and the Y-chromosomal *cynDAZ* cDNA of the Old World monkey *M.fascicularis* and investigated the testicular expression pattern. Comparison with the human *DAZ* genes yielded interesting insights into the evolution of the *DAZ* gene cluster and its role in spermatogenesis.

One of the remarkable features of *cynDAZ* cDNA is the presence of repeat units consisting of exons 7 and 8, whereas in the human *DAZ* cDNA repeat units are mainly formed by exon 7. At the genomic level both exons are present in either species, but transcription of exon 8 in the human is prohibited by mutations affecting the splice sites of exon 8 (6). Taking into account the

observed cDNA structures of *cynDAZ* and *cynDAZL1*, sequence homologies between the different repeats of *cynDAZ* and *DAZ* and the information on the genomic organization of *DAZ* and *DAZL1*, we suggest a revised sequence of evolutionary events leading from a hitherto unknown ancestral *DAZ* gene to the *DAZ* genes present today in the human and the cynomolgus monkey (Fig. 6).

A complete copy of the *DAZL1* transcription unit was transposed from an autosome to the Y chromosome. This translocation must have occurred after the splitting of the Old World and New World monkeys ~40 million years ago (13).

Within the newly transposed gene a 2.4 kb genomic segment encompassing exons 7 and 8 was repeated. The assumption that the same amplification event must have taken place in one lineage is based on the high homology between the junction sequences of the 2.4 kb unit in the human and Old World monkey lineages (Fig. 3). Since the different repeats are not identical in the nucleotide sequences, a pedigree of the origins of the different repeats can be deduced. Amplification of the genomic segment resulted in the hypothetical repeats A and B. A then duplicated again, resulting in A and C, followed by splitting of C, creating D. Four repeats consisting of exons 7 and 8 are now present in the *DAZ* gene.

A deletion in B destroyed the right splice site of exon 8 and a nucleotide substitution in C created a new splice donor site, resulting in a truncated copy of exon 8 (6). These events must have taken place before splitting of species since in both monkey *cynDAZ* and human *DAZ* cDNA the last copy of exon 8 is truncated (35 nucleotides). Repeats A and D remained unaltered.

Splitting of the human and monkey lineages occurred ~20 million years ago. Thereafter a mutation in the left splice site of exon 8 in repeat A disabled this exon in humans and a mutation changed the right splice site of exon 8 in repeat D (6). It appears that most repeats present today in humans are derivatives of repeat D and therefore have the same mutation leading to lack of transcription of exon 8. This deduction is not possible for the monkey since the genomic sequences of the monkey *DAZ* gene are not yet known. In the monkey the last truncated exon 8 is followed by a singular exon 7 (Figs 1 and 6), whereas in the human truncated exon 8 is followed by exon 10. This points to an event disabling also the last copy of exon 8 in the human after the species split. Since these events are presumably specific for the lineage of humans and apes, the cynomolgus monkey still possesses a *DAZ* in which most of the transcribed repeat units consist of exons 7 and 8.

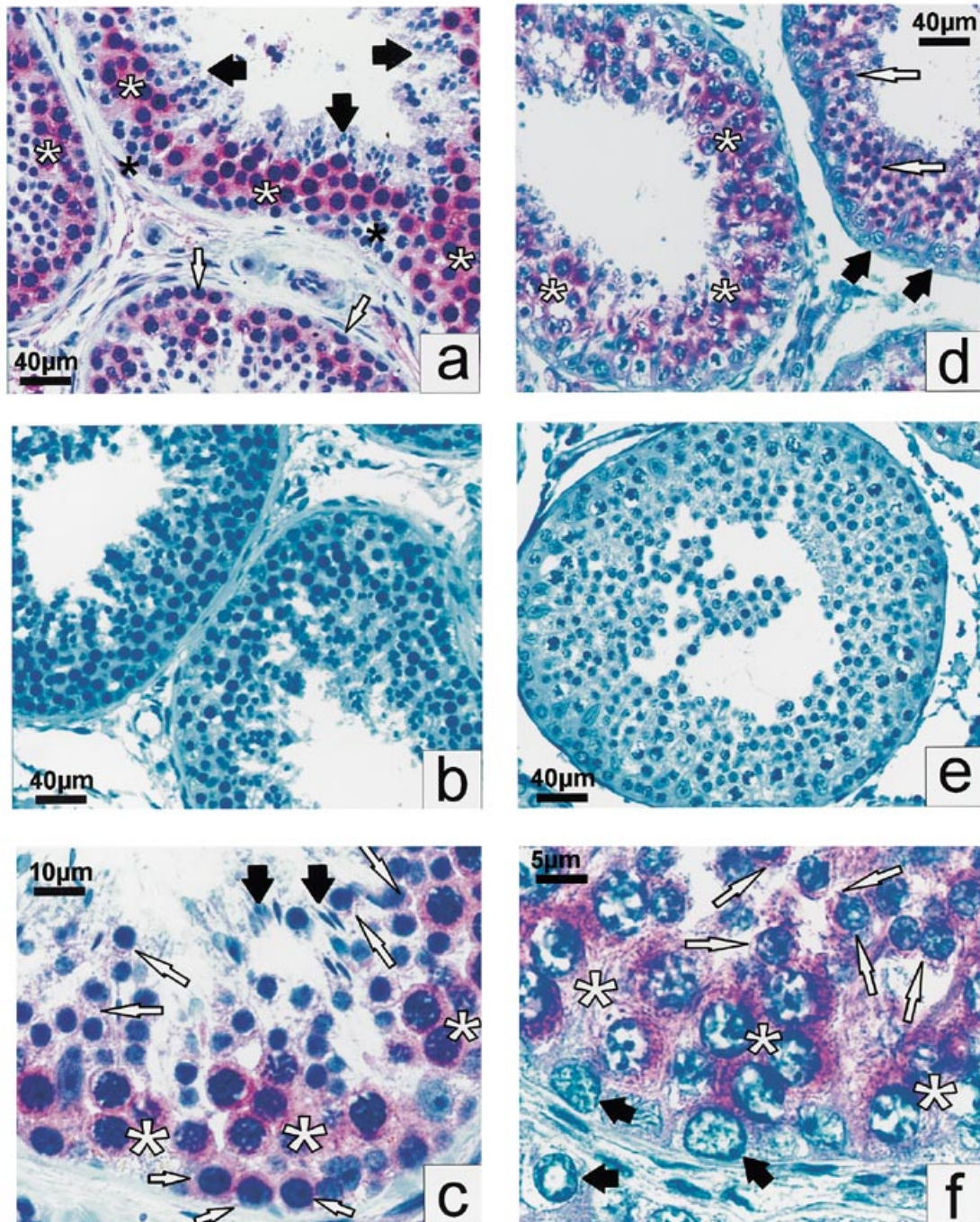


Figure 4. Micrographs from cynomolgus monkey (a–c) and marmoset (d–f) testis stained for *DAZ/DAZLI* using a polyclonal antiserum and APAAP/Neufuchsin (red) for antigen detection and visualization. Sections are counterstained with hematoxylin. (a) Pachytene spermatocytes are stained intensely (white asterisks), whereas early spermatocyte clusters (black asterisks) and spermatids in various phases of elongation (black arrows) are negative. Some spermatogonia are positive (white arrows). (b and e) Preincubation of primary antibody with antigen abolished the stain. (c) High power magnification showing signals in B-type spermatogonia (short white arrows). Round spermatids (long white arrows) are weakly positive whereas elongated spermatids (black arrows) are negative. White asterisks denote clusters of positive pachytene spermatocytes. (d) In the marmoset, pachytene spermatocytes display signal (white asterisks) whereas earlier spermatocytes (black arrows) are negative. White arrows exemplify round spermatids with intense staining of cytoplasmic structures. (f) High power magnification showing that A-type and B-type spermatogonia (black arrows) are negative. White asterisks indicate areas with intensely stained pachytene spermatocytes. Round spermatids (long white arrows) are also positive.

The resulting complete *DAZ* transcript unit then was amplified, giving rise to a multicopy gene family in the human. In Old World monkeys the presence of Y-chromosomal *DAZ* genes has been proven by comparative mapping studies (23) and Southern blot

experiments (7), but the exact determination of *DAZ* copy number is presumably only possible using Fibre-FISH techniques (24). In the cynomolgus monkey at least two copies must have existed, one copy except exon 1 was translocated and inserted into exon 7

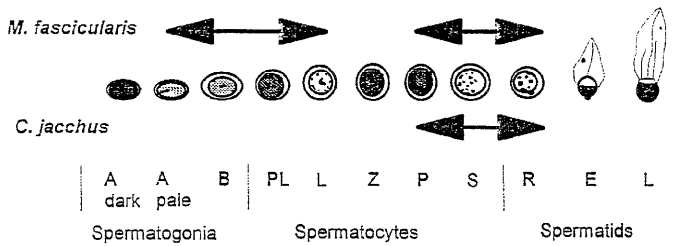


Figure 5. Schematic representation of the *DAZ/DAZLI* protein expression pattern during spermatogenesis in an Old World (*M.fascicularis*) and a New World (*C.jacchus*) monkey. Arrows indicate germ cell types where expression was observed.

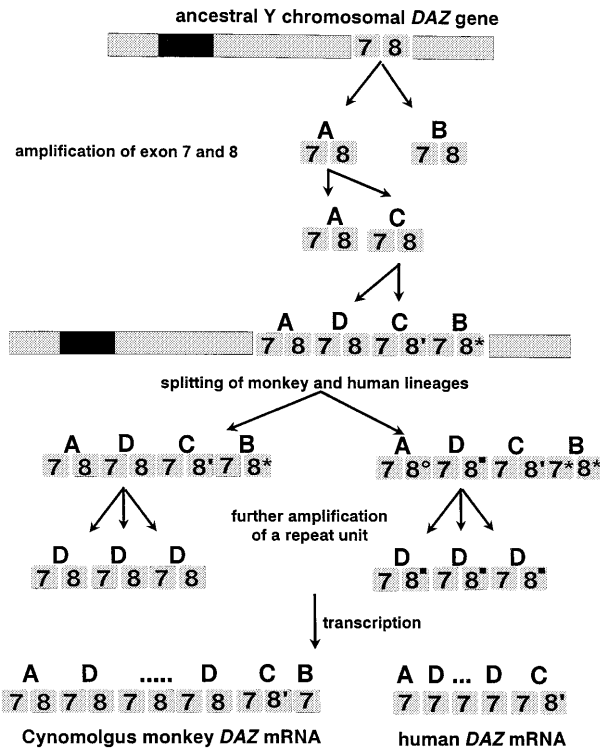


Figure 6. Proposed model for the evolution of *DAZ* in the human and in the cynomolgus monkey. For the evolution of the repeat units a phylogenetic tree was built based on the sequence differences within the different repeat units. Sequence data from monkey and the human were used. The RNA-binding domain of the ancestral Y-chromosomal *DAZ* is indicated by the black box and the remaining ORF, except exons 7 and 8, by the light grey boxes. The different repeats and their origins are given by letters A–D. Mutations affecting the splice sites or truncations of the different exons within a specific repeat are indicated by ° for repeat A, * for repeat B, ' for repeat C and a black square for repeat D. A detailed nucleotide sequence map of the model proposed can be obtained from the authors on request.

of the other gene copy, resulting in the generation of a *cynDAZ* gene as described here.

Northern blot experiments revealed two *cynDAZLI* transcripts with a size of 3.5 and 1.6 kb, respectively. Although the cDNA displays only one typical polyadenylation signal, it is likely that a shorter transcript is generated by alternative polyadenylation. Several *DAZLI* transcripts have also been described in the human

and mouse (2,8). The signal obtained for the 5.8 kb *cynDAZ* transcript is clearly lower when compared with *cynDAZLI* mRNA; however, this does not necessarily reflect an overall decreased level of *cynDAZ* mRNA, since expression could be high in certain germ cells, e.g. spermatogonia, presenting only a minor fraction of the testis homogenates being used for mRNA isolation. The same transcript size is present in three animals, indicating the lack of gross alternative splicing events of *cynDAZ* mRNA.

Since the first description of *DAZ*, the question of whether it is functional or just a vestigial gene cluster on the Y chromosome has been discussed. Along this line substitution rates in the first five exons and four introns of *DAZ* from several species, including humans, were investigated and found not to be significantly different, implying a neutral genetic drift and the absence of functional selective pressures on *DAZ* (25). However, from recent studies (10,24) it is evident that the *DAZ* gene cluster consists of more than two gene copies, as was assumed by Agulnik *et al.* (25), leaving open the possibility that distinct copies of the *DAZ* gene are beneficial for spermatogenesis. For *cynDAZ* it is noteworthy that despite several duplications and rearrangements the ORF is preserved, indicating a selective pressure at the translational level. The view in favor of a function is further strengthened by experiments in which the capacity of *DAZ* to complement the sterile phenotype of the *Da311* null mouse was investigated. Heterologous expression of *DAZ* in these mice increases the number of germ cells and leads to a survival into pachytene stages of meiosis (26). The partial recovery of spermatogenesis in mice by using human *DAZ* also suggests that the target mRNA for *DAZLI* and *DAZ* during spermatogenesis is either similar or identical. Studies in *Xenopus* have shown that the *DAZLI* homolog interacts with G- and U-rich RNAs *in vivo* (16). In *Drosophila Boule*, a ortholog of *DAZLI*, interacts with the translational regulation of *twine*, encoding a Cdc25-type phosphatase, which is crucial for the transition from G₂ to M phase during meiosis (27). In this regard the presence of 12 phosphorylation motifs is an interesting feature of *cynDAZ*. If these sites undergo phosphorylation it could have an impact on the activity of *cynDAZ*. Studies in yeast indicated that RNA-binding proteins control the cell cycle switch from mitotic to meiotic cell divisions (28). The protein is phosphorylated by protein kinases, thereby promoting the mitotic cycle or triggering the meiotic cycle by dephosphorylation. This model would in part resemble the expression pattern of *DAZ/DAZLI* that is present in mitotic (29) and meiotic (17,18,30) cells.

The protein expression of *DAZLI* and *DAZ* is confined to certain germ cell types, but seems to vary between species. In rodents, a large expression window for *DAZLI*, ranging from intermediate spermatogonia to round spermatids, exists (18). This window is much narrower in New World monkeys, where expression is restricted to late spermatocytes and early spermatids. In Old World monkeys *cynDAZ/DAZLI* protein could be detected during spermatogonial stages and advanced pachytene spermatocytes (Fig. 5). Although in our immunohistochemical approach it was not possible to distinguish between *cynDAZ* and *cynDAZLI*, it is tempting to speculate that the expression pattern obtained in spermatogonia might represent *cynDAZ*. These findings point to an additional function of *cynDAZ/cynDAZLI* during early stages of spermatogenesis in the Old World monkey not needed in the New World monkey. Interestingly, *DAZ* expression is highest in human spermatogonia (17) and deletions

of *DAZ* in infertile men frequently cause a Sertoli-cell-only syndrome or oligozoospermia, strongly indicating that *DAZ* is an essential factor for the generation and maintenance of spermatogonial stem cell population.

Finally, the fact that *cynDAZ* possesses two RNA recognition motifs (RRMs) might point to a function different from *DAZ* or *DAZLI* homologs. The presence of two RRM motifs can either affect binding affinities to target mRNAs or change the type of target mRNA. Similar to *cynDAZ*, a number of RNA-binding proteins have been described bearing two or more RRM motifs, all being essential for spermatogenesis (31). In nearly all cases the target mRNA is not known, and further research is needed to identify target mRNAs for *cynDAZ* and *DAZ*, and gain insights into the functional role of the *DAZ* gene family during spermatogenesis.

MATERIALS AND METHODS

Animals

Testes from adult cynomolgus monkeys (*M.fascicularis*) and common marmosets (*C.jacchus*) were collected at autopsy and partly snap-frozen or fixed in Bouin's fluid for subsequent analysis.

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. Plating of recombinant phages, generation of replica filters and hybridization were performed according to the standard protocols. Hybridization was performed at 60°C for 16–18 h. Filters were washed at a final stringency of 1× SSC/0.1% 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.

5'-RACE of *cynDAZ*

Based on the partial cDNA sequence a specific *cynDAZ* reverse primer (GCGCGACTCGGGCTGAAGATATG; position 449–472) was used for cDNA synthesis from monkey testis RNA. Subsequent PCR was performed using a forward primer (GCCTGCCACCACCATGTCTGC, position –13 to 8 based on the human *DAZ* sequence (2)). The PCR fragments obtained were cloned into the TA cloning vector (Invitrogen, Heidelberg, Germany) and sequenced.

Northern blot hybridization

mRNA was isolated from different tissues of cynomolgus monkeys using the Fast Track kit (Invitrogen, Heidelberg, Germany). Electrophoresis of mRNA was performed in 1% denaturing agarose gels, the mRNA was blotted onto nylon membranes (Amersham, Freiburg, Germany) and fixed by crosslinking through UV irradiation. Filters were prehybridized at 58°C in 5× SSPE (1× SSPE: 15 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA), 50% formamide, 2× Denhardt's solution and 1% SDS with 0.1 mg/ml denatured salmon sperm DNA. Hybridization

conditions were identical to those for prehybridization but with the addition of a [³²P]CTP-labelled cRNA probe with a final concentration of 0.5 × 10⁶ c.p.m./ml. As probe either a cDNA cloned in pGEM-T (Promega, Madison, WI) containing the ORF of *cynDAZLI*, linearized by *SacII* digestion and transcribed by T7-RNA polymerase or a cDNA containing four exon 7 and 8 repeat units from *cynDAZ*, linearized by *SalI* digestion and transcribed by T3-RNA polymerase, were used. Hybridization was performed overnight for 16–18 h followed by washing at a final stringency of 0.1 SSC/0.1 SDS at 68°C. The blots were exposed to X-ray films (Amersham) for 2–7 days using intensifying screens at –80°C.

Genomic DNA isolation

Genomic DNA was isolated from blood samples of women, men and cynomolgus monkeys using the nucleon extraction and purification kit (Amersham). In the different PCR experiments 100–300 ng DNA were used (PCR-cycling conditions: 35× 40 s at 94°C, 30 s at 58°C, 1.5 min at 72°C). For the amplification of the 2.4 kb genomic unit junctions in the monkey and human two primers based on the human sequence were used: forward, CTCTGCCTCTGGCTTTACCA; reverse, GAGGAGGCATCTGGAAATCATT.

Sequence analysis

For sequence analysis the FASTA (version 3) program was used. The phylogenetic studies were performed with the PHYLIP (Phylogeny Inference Package, version 3.5c) program.

Immunohistochemistry

Bouin's fixed specimens were dehydrated, embedded in paraffin, and sectioned at 5 µm. After deparaffinization, rehydration and blocking of unspecific binding using 5% normal porcine serum, the sections were incubated with a rabbit polyclonal antibody (antiserum 149) directed to a peptide (HGKKLKLGPAIRKQKL) common in human *DAZ* and *DAZLI* (R. Reijo *et al.*, unpublished data) at 1:400–1:1000 dilutions overnight at 4°C. Following washing steps [3× TBS (0.05 M Tris, 0.15 M NaCl, pH 7.6), 5 min each], sections were incubated with a second antibody (monoclonal mouse anti-rabbit Ig, 1:50 dilution; Dako, Hamburg, Germany) for 30 min at room temperature. After a second washing step an antibody–enzyme complex (porcine APAAP; Sigma, Deisenhofen, Germany) was added and incubated for 30 min at room temperature. After a final washing step the sections were incubated for 15 min with Newfuchsin. The reaction was stopped, sections were counterstained with haematoxylin, dehydrated and mounted. For assessment of method specificity the primary antibody was omitted. Antibody specificity was evaluated by pre-incubation of the primary antibody overnight at 4°C using the synthetic antigenic peptide. For the experiments, antibody dilution was kept constant at 1:400 and a 0.1% peptide (1 mg/ml) solution was added at volumes ranging from 1:1, 2:1, 5:1 and 8:1 (peptide:antibody). On the next day, following washing steps, the immunohistochemical protocol was applied as described above, using the peptide/antibody mixture as primary antibody solution.

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REFERENCES

- Tiepolo, L. and Zuffardi, O. (1976) Localization of factors controlling spermatogenesis in the non-fluorescent portion of the human Y chromosome long arm. *Hum. Genet.*, **34**, 119–124.
- Reijo, R., Lee, T.Y., Salo, P., Alagappan, R., Brown, L.G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., de la Chapelle, A., Silber, S. and Page, D.C. (1995) Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nature Genet.*, **10**, 383–393.
- Vogt, P., Edelmann, A., Kirch, S., Henegariu, O., Hirschmann, P., Kiesewetter, F., Köhn, F.M., Schill, W.B., Farah, S., Ramos, C., Hartmann, M., Hartschuh, W., Meschede, D., Behre, H.M., Castel, A., Nieschlag, E., Weidner, W., Gröne, H.J., Engel, W. and Haidl, G. (1996) Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum. Mol. Genet.*, **5**, 933–943.
- Simoni, M., Gromoll, J., Dworniczak, B., Rolf, C., Abshagen, K., Kamischke, A., Carani, C., Meschede, D., Behre, H.M., Horst, J. and Nieschlag, E. (1997) Screening for deletion of Y chromosome involving the *DAZ* (Deleted in Azoospermia) gene in azoospermia and severe oligozoospermia. *Fertil. Steril.*, **67**, 542–547.
- Vogt, P. (1998) Human chromosome deletions in Yq11, AZF candidates and male infertility: history and update. *Mol. Hum. Reprod.*, **4**, 739–744.
- Saxena, R., Brown, L.G., Hawkins, T., Alagappan, R.K., Skaletsky, H., Reeve, M.P., Reijo, R., Rozen, S., Dinulos, M.B., Distche, C.M. and Page, D.C. (1996) The *DAZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transposed, repeatedly amplified and pruned. *Nature Genet.*, **14**, 292–299.
- Shan, Z., Hirschmann, P., Seebacher, T., Edelmann, A., Jauch, A., Morell, J., Urbitsch, P. and Vogt, H.P. (1996) A SPGY copy homologous to the mouse gene *DAZL1a* and the *Drosophila* gene *boule* is autosomal and expressed only in human male gonad. *Hum. Mol. Genet.*, **5**, 2005–2011.
- Yen, P.H., Chai, N.N. and Salido, E.C. (1996) The human autosomal gene *DAZL1A*: testis specificity and a candidate for male infertility. *Hum. Mol. Genet.*, **5**, 2013–2017.
- Seboun, E., Barboux, S., Bourgeron, T., Nishi, S., Agulnik, A., Egashira, M., Nikkawa, N., Bishop, C., Fellous, M., McElreavy, K. and Kasahara, M. (1997) Gene sequence, localization, and evolutionary conservation of *DAZL1A*, a candidate male sterility gene. *Genomics*, **41**, 227–235.
- Yen, P.H., Chai, N.N. and Salido, E.C. (1997) The human *DAZ* genes, a putative male infertility factor on the Y chromosome, are highly polymorphic in the *DAZ* repeat regions. *Mamm. Genome*, **8**, 756–759.
- Carani, C., Gromoll, J., Brinkworth, M.H., Simoni, M., Weinbauer, G.F. and Nieschlag, E. (1997) *cynDAZL1A*: a cynomolgus homologue of the human autosomal *DAZ* gene. *Mol. Hum. Reprod.*, **3**, 479–483.
- Gromoll, J., Simoni, M., Weinbauer, G.F. and Nieschlag, E. (1998) Spermatogenesis-specific genes deleted in infertile men: *DAZ/DAZH* clinical aspects and animal models. In Stefanini, M., Boitani, C. and Galdieri, M. (eds), *Testicular Function: From Gene Expression to Genetic Manipulation*. Springer Verlag, Heidelberg, pp. 273–294.
- Kumar, S. and Hedges, B. (1998) A molecular timescale for vertebrate evolution. *Nature*, **392**, 917–920.
- Cooke, H.J., Lee, M., Kerr, S. and Ruggiu, M. (1996) A murine homologue of the human *DAZ* gene is autosomal and expressed only in male and female gonads. *Hum. Mol. Genet.*, **5**, 513–516.
- Reijo, R., Seligman, J., Dinulos, M.B., Jaffe, T., Brown, L.G., Distche, C.M. and Page, D.C. (1996) Mouse autosomal homolog of *DAZ*, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. *Genomics*, **35**, 346–352.
- Houston, D.W., Zhang, J., Maines, J.Z., Wasserman, S.A. and King, M.L. (1998) A *Xenopus* *DAZ*-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophila boule*. *Development*, **125**, 171–180.
- Menke, D.B., Mutter, G.L. and Page, D.C. (1997) Expression of *DAZ*, an Azoospermia Factor candidate, in human spermatogonia. *Am. J. Hum. Genet.*, **60**, 237–241.
- Ruggiu, M., Speed, R., Taggart, M., McKay, S.J., Kilanowski, F., Saunders, P., Dorin, J. and Cooke, H.J. (1997) The mouse *DAZL1a* gene encodes a cytoplasmic protein essential for gametogenesis. *Nature*, **389**, 73–77.
- Eberhart, C.G., Maines, J.Z. and Wasserman, S.A. (1996) Meiotic cell cycle requirement for a fly homologue of human Deleted in Azoospermia. *Nature*, **381**, 783–785.
- Weinbauer, G.F. and Nieschlag, E. (1999) Testicular physiology of primates. In Weinbauer, G.F. and Korte, R. (eds), *Reproduction in Nonhuman Primates: A Model System for Human Reproductive Physiology and Toxicology*. Waxmann Verlag, Münster/New York, pp. 13–26.
- Weinbauer, G.F. and Nieschlag, E. (1998) The role of testosterone in spermatogenesis. In Nieschlag, E. and Behre, H.M. (eds), *Testosterone: Action, Deficiency, Substitution*. 2nd edn. Springer Verlag, Heidelberg, pp. 143–168.
- Zhengwei, Y., McLachlan, R.I., Bremner, W.J. and Wreford, N.G. (1997) Quantitative (stereological) study of the normal spermatogenesis in the adult monkey (*Macaca fascicularis*). *J. Androl.*, **8**, 681–687.
- Gläser, B., Grütznher, F., Willmann, U., Stanyon, R., Arnold, N., Taylor, K., Rietschel, W., Zeitler, S., Toder, R. and Schempp, W. (1998) Simian Y chromosomes: species-specific rearrangements of *DAZ*, *RBM*, and *TSPY* versus contiguity of *PAR* and *SRY*. *Mamm. Genome*, **9**, 226–231.
- Gläser, B., Yen, P.H. and Schempp, W. (1998) Fibre-FISH unravels apparently seven *DAZ* genes or pseudogenes clustered within a Y-chromosome region frequently deleted in azoospermic males. *Chromosome Res.*, **6**, 481–486.
- Agulnik, A.I., Zharkikh, A., Boettger-Tong, H., Bourgon, T., McElreavy, K. and Bishop, C.E. (1998) Evolution of the *DAZ* gene family suggests that Y-linked *DAZ* plays little, or a limited, role in spermatogenesis but underlines a recent African origin for human populations. *Hum. Mol. Genet.*, **9**, 1371–1377.
- Slee, R., Grimes, B., Speed, R.M., Taggart, M., Maguire, S.M., Ross, A., McGill, N.I., Saunders, P.T.K. and Cooke, H.J. (1999) A human *DAZ* transgene confers partial rescue of the mouse *DAZ1* null phenotype. *Proc. Natl. Acad. Sci. USA*, **14**, 8040–8045.
- Maines, J.Z. and Wasserman, S.A. (1999) Post-transcriptional regulation of the meiotic Cdc25 protein Twine by the *DAZ1* orthologue *Boule*. *Nature Cell Biol.*, **1**, 171–174.
- Watanabe, Y., Shinozaki-Yabana, S., Chikashige, Y., Hiraoka, Y. and Yamamoto, M. (1997) Phosphorylation of RNA-binding protein controls cell cycle switch from mitotic to meiotic in fission yeast. *Nature*, **386**, 187–190.
- Niederberger, C., Agulnik, A.I., Cho, Y., Lamb, D. and Bishop, C.E. (1997) *In situ* hybridization shows that *DAZL1a* expression in mouse testis is restricted to premeiotic stages IV–VI of spermatogenesis. *Mamm. Genome*, **7**, 277–278.
- Seligman, J. and Page, D.C. (1998) The *DAZ* gene is expressed in male and female embryonic gonads before germ cell sex differentiation. *Biochem. Biophys. Res. Commun.*, **245**, 878–882.
- Venables, J.P. and Eperon, I.C. (1999) The roles of RNA-binding proteins in spermatogenesis and male infertility. *Curr. Opin. Genet. Dev.*, **9**, 346–354.