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At the Cutting Edge

Genomic heterogeneity and instability of the AZF locus on the human Y chromosome

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Abstract

The spermatogenesis locus azoospermia factor (AZF) in Yq11 has been mapped to three microdeletion intervals designated as AZFa, AZFb, and AZFc. They are caused by intrachromosomal recombination events between large homologous repetitive sequence blocks, and AZFc microdeletions are now recognised as the most frequent known genetic lesion causing male infertility. However, in the same Y-region, large genomic heterogeneities are also observed in fertile men, and only complete AZFa and AZFb deletions are associated with a specific testicular pathology. Partial AZF deletions are associated with variable pathologies and partial AZFc deletions may even have no impact on male fertility. This suggests a genetic redundancy of the multi-copy genes in AZFb and AZFc and a causative relationship between the occurrence of first microdeletions then macrodeletions in the repetitive structure of Yq11 where large palindromes are probably promoting multiple gene conversions and AZF rearrangements.

Keywords: Y genomic heterogeneity; AZF locus and spermatogenesis function; Y-haplogroups and AZFc plasticity; AZFc deletions and mosaic karyotypes

1. Introduction

Novel findings concerning the genomic heterogeneity of the human Y chromosome have recently been reported by several research groups to occur in Yq11 (Bosch and Jobling, 2003; Repping et al., 2003; Vogt and Fernandes, 2003; Fernandes et al., 2004). They have inevitably led to new speculations about the evolutionary future of this male-specific chromosome (Jobling and Tyler-Smith, 2003) but also to an increased understanding of the azoospermia factor (AZF) locus in Yq11 concerning its function in human spermatogenesis. AZF was first mapped by cytogenetic observations of microscopically visible deletions (i.e. macrodeletions) and rearrangements of the Y chromosome in sterile patients, which always affected Yq11 (Neu et al., 1973, Tiepolo and Zuffardi, 1976). Monocentric Yq-chromosomes, dicentric isochromosomes of the short arm (dic-Yp), Y chromosomes with a ring structure (ring-Y), or translocation of the Y to an autosome or the X chromosome, all characterised by a complete absence of the fluorescent Y heterochromatin in distal Yq (Yq12) were most often reported (Sandberg, 1985; Vogt, 1996). However, for a long time nobody believed in a functional AZF locus because genomic heterogeneity of the Y long arm was also observed in fertile men and it seemed impossible to distinguish those variabilities in order to select for those which might disrupt an essential Y spermatogenesis gene.

2. Molecular AZF deletion analysis maps AZFa, AZFb and AZFc in Yq11

New hope for mapping the proposed *AZF* gene(s) arose after the detection of microdeletions in Yq11. In PCR-multiplex assays genomic DNA samples from 370 infertile men with a normal karyotype (46,XY) were screened for the deletion of Y-specific DNA loci (sequence tagged sites (STS)) mapped

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to a linear interval map in Yq11 (Vollrath et al., 1992; Vogt et al., 1996). Three microdeletions with different locations in Yq11 were detected (Vogt et al., 1996; Vogt, 1998), and as each microdeletion occured "de novo" (i.e., was restricted to the patient's Y chromosome) and was associated with the occurrence of a specific testicular pathology (Fig. 1A), it was proposed that not only one, but three distinct AZF loci (AZFa, AZFb, AZFc) were present in Yq11. The frequency of these AZF deletions in infertile men ranges in worldwide surveys from 5 to 20% (Vogt, 1998; Krausz and McElreavey, 1999; Krausz et al., 2003). They therefore became the most frequent known genetic lesion causing male infertility.

However, common doubts on their functional AZF aspects came to life again after the PCR based STS interval maps in Yq11 seemed not to fuse to a general reference map (Vogt et al., 1997). In different laboratories STS deletions in Yq11 were mapped occasionally to multiple sites and the occurrence of non-contiguous deletion spots in the same Y region suggested a variable location of at least some of them (Najmabadi et al., 1996; Stuppia et al., 1996a; Foresta et al., 1997). A general order of these anonymous STS loci in Yq11 seemed not to exist and since single familial STS deletions were also found in fertile men (Kent-First et al., 1996; Pryor et al., 1997; Vogt, 1998) again a big question mark hung over the ability of these genomic STS deletion maps to reveal a genetic lesion of an AZF spermatogenesis gene responsible for the patients' infertility. Some help was offered from the first laboratory guidelines suggesting the use of a common robust set of six non-repetitive and non-polymorphic STS loci and appropriate controls to provide quality control of the AZF-microdeletion analysis in different laboratories, and also to optimise the PCR multiplex protocol (Simoni et al., 1999; Simoni, 2001). However, the molecular extensions of the AZF microdeletions and its complete gene content remained first largely unknown fostering furtheron some doubts on the presence of a distinct *AZF* gene in Yq11.

3. AZF deletions in Yq11 are caused by intrachromosomal recombination events

Today, we know that AZFa, AZFb and AZFc deletions are caused by intrachromosomal recombination events between large homologous repetitive sequence blocks located in Yq11 (Kamp et al., 2000; Sun et al., 2000; Kuroda-Kawaguchi et al., 2001; Repping et al., 2002). "Complete AZF microdeletions", being indeed a causative agent of the patient's infertility, can now be easily distinguished from suspected polymorphic or partial AZF deletions by simply estimating the locations of the patients' breakpoint-fusion regions in Yq11 (Fig. 1B). These should always be found in a specific fused repetitive sequence block, for AZFa in HERV15yq1/yq2 (Kamp et al., 2000), for AZFb in the P5/proximal P1 repeat blocks (Repping et al., 2002), and for AZFc in the b2/b4 repeat blocks (Kuroda-Kawaguchi et al., 2001) (Fig. 1B). Interestingly, AZFb + c deletions are not AZFb plus AZFc deletions but were created by a different intrachromosomal recombination event between P5 or P4 and the distal P1 repeat blocks and both were overlapping with AZFc deletions (Fig. 1B). For the clinically most important diagnosis of *"complete AZF deletions"* causing a specific testicular pathology, a novel set of STS loci was established mapping around the different AZF breakpoint-fusion regions. They identify the completeness of each AZF deletion by a simple deletion pattern (Fig. 1B). Recurrently, partial and polymorphic AZF deletions are reported as well, in AZFa (Qureshi et al., 1996; Kamp et al., 2000; Blanco et al., 2000), in AZFb (Ferlin et al., 2003; Prosser et al., 1996) and in AZFc (Stuppia et al., 1996b; Jobling et al., 1996; Repping et al., 2003; Fernandes et al., 2002; 2004). Therefore, if one wants to be sure that the patient's AZF deletion indeed has caused his testicular pathology an estimation of the extension of his AZF deletion seemed to be strongly recommended (Krausz et al., 2000, 2003).

4. There is no unique Y chromosome sequence in human populations

After the complete sequence of the human Y chromosome had been published (Tilford et al., 2001; Skaletsky et al., 2003), worries about the functional consequences of a diagnosed AZF-deletion for the patient's infertility rose, however, up again, concerning especially the plasticity of the homologous large repetitive sequence blocks (also coined "amplicons") in distal Yq11. They make up almost all of the AZFc sequence and 50% of the AZFb sequence, respectively (Fig. 1C). As first suggested by Yen (2001), distinct intrachromosomal recombinations between homologous amplicons in AZFb and AZFc should cause different partial AZFb and AZFc deletions because most of them are organised in the same polarity. Therefore, no unique AZFc sequence amplicon order is expected in men and the AZFc sequence now published in GenBank might be not present in all populations. It would then be most interesting to know which of them are restricted to men with a phenotype of infertility as found for the complete AZFa, AZFb, and AZFc deletions, and which are also found in men with normal fertility and with a normal sperm count.

A partial and polymorphic deletion of 1.6 Mb in AZFc persisting over many generations was detected by Repping et al. (2003). They were designated as "green–red/green–red" ("gr/gr") AZFc deletions (Fig. 2A) as the Fiber-FISH method used for their analyses could not distinguish deletions caused by g1/g2 recombinations from those caused by r1/r3 or r2/r4 recombinations (Fig. 2A). The authors observed a higher frequency of gr/gr deletions in men with some spermatogenic failure, suggesting an increased risk for the occurrence of infertility in these men (Repping et al., 2003). This risk might be restricted to the subgroup of men with g1/g2 recombinations in AZFc (Fig. 2A) recently found only in some men with severe oligozoospermia (Fernandes et al., 2002).

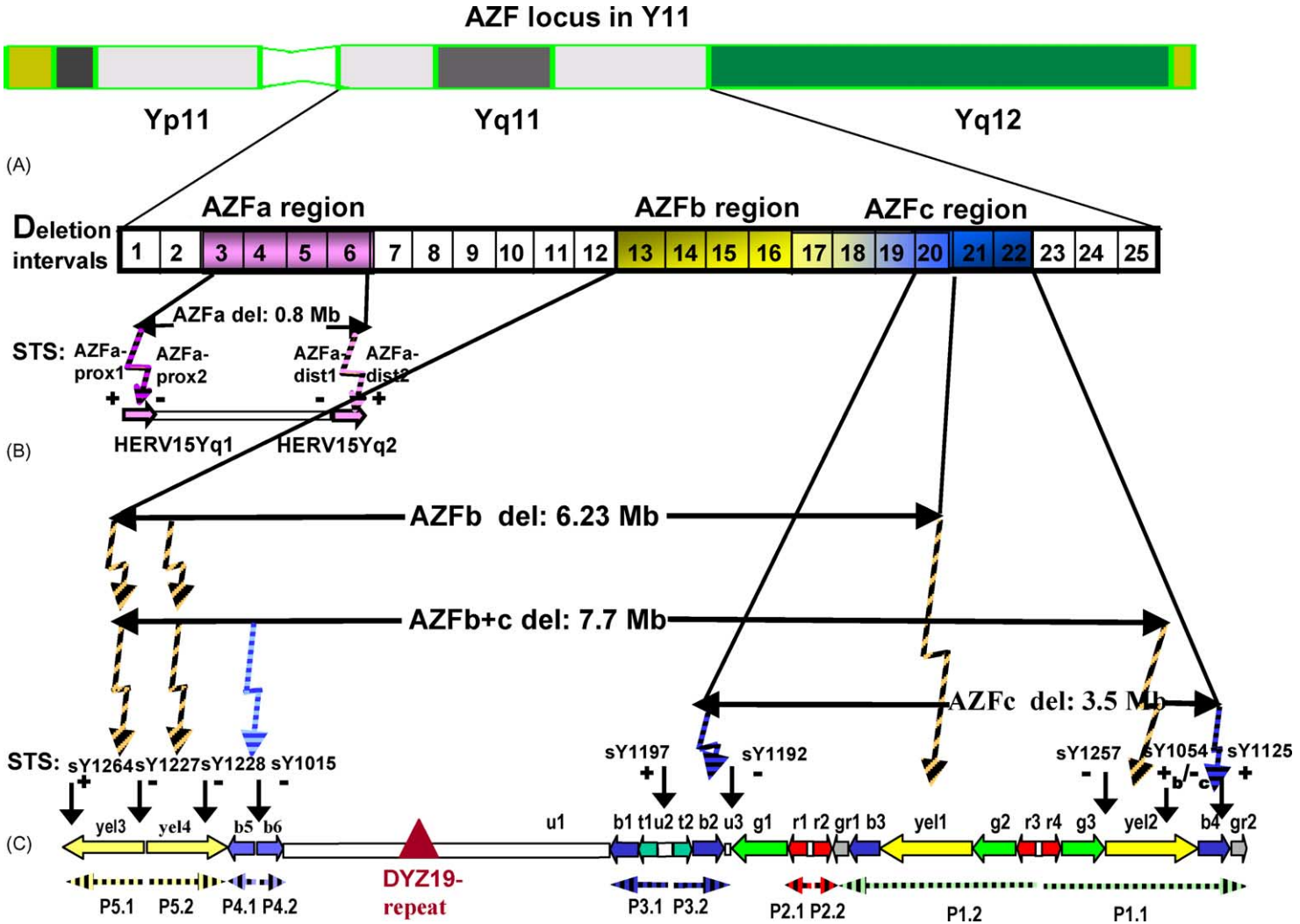

Fig. 1. Schematic view of the AZF locus in Yq11. (A) The AZF locus was first subdivided by molecular deletion analyses into three regions of the deletion map of Vogt et al. (1996), which divided Yq11 into 25 intervals (D1–D25). (B) Complete AZFa deletions, associated always with a complete Sertoli-cell-only- (SCO) syndrome, are caused by recombination of two homologous HERV15Yq1/q2 blocks located in proximal Yq11. Complete AZFb deletions, associated always with meiotic arrest, overlap with AZFc deletions as indicated and are caused by recombination between large homologous repetitive sequence blocks also coined amplicons (Kuroda-Kawaguchi et al., 2001; Repping et al., 2002). According to the colour code of Kuroda-Kawaguchi et al. (2001) they are designated as P1.1 and P1.2 (yel1 and yel2) in the P1 palindrome and P5.1 and P5.2 (yel3 and yel4) in the P5 palindrome, respectively. Similarly, AZFc deletions are caused by recombination between the blue amplicons b2 and b4. AZFc deletions show a mixed atrophy of germ cells in the patients' testis tubules and hypospermatogenesis. In patients with AZFb + c deletion, the distal breakpoint was found to be not in the b4 amplicon but in the yellow amplicon yel2 (Repping et al., 2002). The STSs which can be used to diagnose complete AZFa, AZFb, or AZFc deletions with simple duplex PCR experiments are given above each amplicon; "−" means absence of this marker, "+" means presence of this marker when the corresponding AZF deletion is complete. STS sY1054 is "+" in patients with complete AZFb or AZFb + c deletion but "−" in all patients with an AZFc deletion. For more details of the corresponding PCR-experiments see the original papers: AZFa: Kamp et al. (2001); AZFb, AZFb + c: Repping et al. (2002); AZFc: Kuroda-Kawaguchi et al. (2001). (C) The five possible palindromic structures P1–P5 and the DYZ19 repeat in AZFb are marked with the extensions of both arms below the amplicon structure in a similar colour code as that used for the corresponding amplicons.

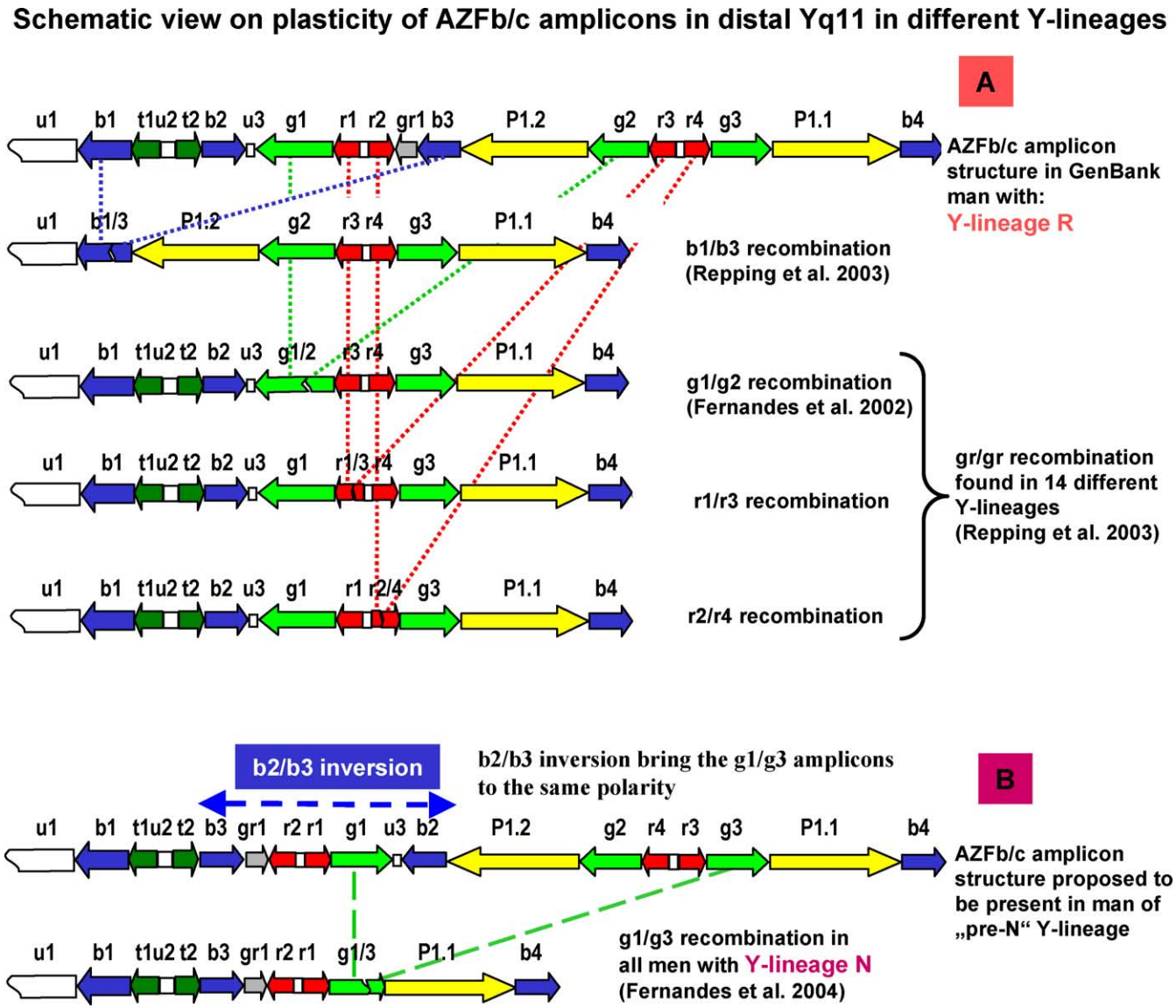

Fig. 2. Schematic view of the plasticity of the AZFb/c amplicons in distal Yq11 in different Y-lineages named according to The Y Chromosome Consortium (2002). (A) The Y haplogroup R structure (top) and four different products (below) arising from it by homologous recombination between amplicons organised in the same polarity, as predicted by Yen (2001). One infertile man with a b1/b3 recombined AZFb/c structure was found by Repping et al. (2003), and five infertile men with a g1/g2 recombined AZFc structure were found by Fernandes et al. (2002). Men with an AZFc structure recombined between the r1/r3 or r2/r4 amplicons have not yet been described but are further subgroups of the men with a gr/gr recombined structure (Repping et al., 2003) found in 14 different Y-lineages. (B) A g1/g3 recombined AZFc structure was proposed in all men with Y-lineage N independent of their fertility status (Fernandes et al., 2004). To organise these green amplicons in the same polarity, an inversion between the b2/b3 amplicon block is needed to occur in the “pre-NY” chromosome (top).

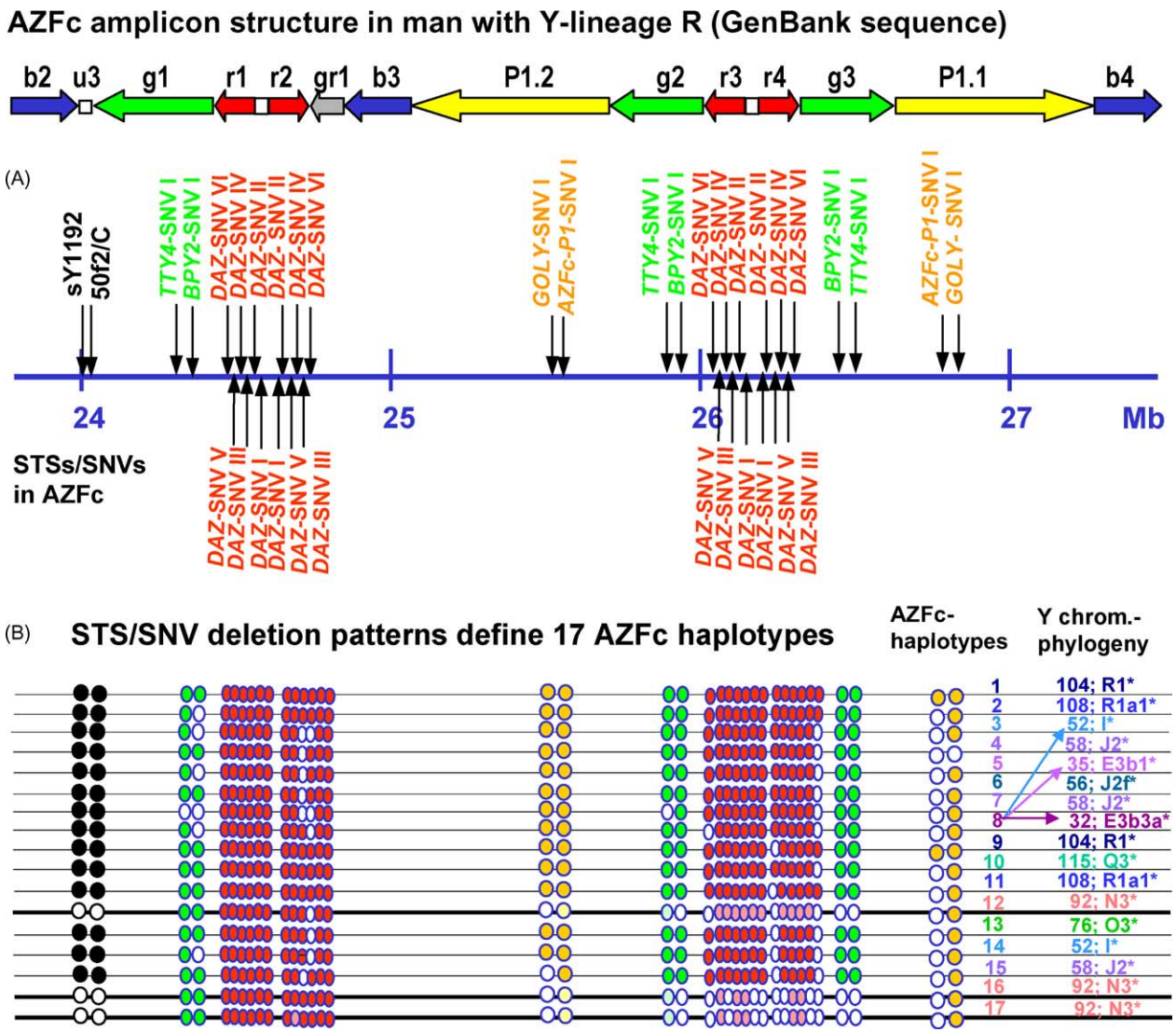

Fig. 3. (A) Schematic view of the AZFc amplicon structure in Y-lineage R, and the location of a series of STSs/SNVs along 3 Mb of the corresponding GenBank sequence between 23.79 and 27.49 Mb. (B) Seventeen different patterns of presence (filled circle) and absence (empty circle) of an STS or SNV in the AZFc sequence were identified (Fernandes et al., 2004). They were associated with 10 different Y chromosomal haplogroups as indicated on the right with the specific codes of Underhill et al. (2000) and The Y Chromosome Consortium (2002) marked in different colours. Eight Y-lineages are known to be common in Europe; the others (O3* and Q3*) are common in the countries of origin of these individuals (China and Costa Rica, respectively). AZFc haplotype 8 was found in three different Y-lineages all belonging to group III of Underhill et al. (2000). Five Y-lineages, I*, J2*, N3*, R1*, R1a1*, were associated with multiple AZFc haplotypes. Thick lines represent the AZFc haplotypes 12, 16, 17 all belonging to Y-lineage N. The pale colour of some circles on these lines indicates that these multi-locus SNVs were inferred to be absent because of all unambiguously typed flanking and intervening markers were absent. For further details see text and Fernandes et al. (2004).

However, since the other gr/gr subgroups are not yet identified in Y chromosomes from either fertile or infertile individuals any influence of also r1/r3 or r2/r4 recombinations on male fertility can not be excluded.

A second partial AZFc deletion including the *DAZ3/DAZ4* genes in distal AZFc and the AZFc-u3 block in proximal Yq11 was recently found by Fernandes et al. (2004). This AZFc deletion became visible by Southern blots using the probes 49f and 50f2. The *DAZ* DNA probe 49f is known to mark on Southern blots the presence or absence of each individual *DAZ* gene copy after restriction with *Eco*RV and *Taq*I and of the gene doublets *DAZ1/DAZ2* and *DAZ3*/*DAZ4* after restriction with *Sfi*I (Fernandes et al., 2002, 2004). Using this probe, the deletion of a large DNA fragment (∼550 kb) in distal AZFc including the complete *DAZ3*/*DAZ4* genes could be shown experimentally (Fernandes et al., 2004).

Using an extensive binary marker set, 18 Y-chromosomal haplogroups designated "A-R" were now defined in the first common Y-chromosomal phylogenetic tree (The Y Chromosome Consortium, 2002). With this marker system, the AZFc sequence published in GenBank was found to originate from the Y chromosome of a man with Y haplogroup R and the Y chromosome with deletion of the AZFc-u3 block and *DAZ3*/*DAZ4* from Y lineage N (Fernandes et al., 2004). Both Y chromosomal haplogroups probably diverged 36,000 years ago (Hammer and Zegura, 2002) and can therefore be considered both to have no detrimental effect on men's fertility. Indeed Y lineage N has dispersed successfully throughout northern Europe and Asia making up ∼12% of Y chromosomes in one world-wide survey and forming the majority in some populations such as the Finns (∼52%) and Yakuts (Sakha; 86%) (Zerjal et al., 1997). Partial AZFc deletions based on gr/gr recombinations have arisen independently on 14 different Y-lineages (Repping et al., 2003) and although their frequency was found to be higher in men with some spermatogenic failure it is still unknown whether this association is true in all Y-lineages.

Most interesting, single deletions of the 50f2 locus in the proximal AZFc-u3 block (50f2/C: GDB accession no. 168024) were already found earlier on the Y chromosome of men from 46 different populations (Jobling et al., 1996). Some 50f2/C deletions were found to extend to the neighbouring *RBMY* gene copies (in b1 and b2 amplicons). In summary, six independent deletion events and four duplications affecting 50f2/C were identified, together representing almost 8% of normal men (Jobling et al., 1996). Although all these 50f2/C deletions are reported to include no *DAZ* gene deletions, as now was found in all men from Y lineage N, one of the deletions described in 1996, the haplogroup 12 "small" 50f2/C deletion might be considered as identical.

The gr/gr deletions were identified on the background of the AZFc GenBank amplicon order. The partial AZFc deletions analysed in men from Y lineage N would not be possible on this structure by a single recombination event because both gaps (AZFc-u3 and *DAZ3/DAZ4*) are separated by 2Mb genomic DNA (Fig. 3A). Therefore, inversion of the b2/b3 amplicons in the AZFc GenBank sequence were predicted for men from the "pre-N" lineage before a g1/g3 recombination event could delete both, AZFc-u3 and *DAZ3/DAZ4* (Fig. 2B). Unfortunately, the AZFc amplicon structure of a man from a "pre-N" Y lineage is not yet defined. It might be found in men of Y lineage O; a sister clade to N in the current Y phylogeny (Jobling and Tyler-Smith, 2003). Other inversion events in the AZFc sequence also leading to the AZFc amplicon structure of Y lineage N can therefore not yet excluded.

They can be predicted from the amplicon structure in the AZFc GenBank sequence because of the general potential of hairpin formations between the inverted homologous amplicons (Figs. 1 and 2). In distal AZFc these might include the complete P1 palindrome structure extending from the ends of b3–b4 or extending from the ends of P1.1/P1.2 or g2/g3, or r3/r4, respectively. Similarly, in distal AZFb and proximal AZFc we can expect b1/b2, t1/t2, b2/b3, g1/g3, and r1/r2 inversions. With view to the amplicon structure in AZFc of Y lineage N an alternative mechanism for its construction might therefore be also an inversion of the g1/g3 amplicons in an "pre-N" Y-lineage with a subsequent b2/b3 recombination event.

5. AZFc sequence plasticity

The plasticity of the AZFc sequence in different Y chromosomal haplogroups was also explored by using a series of STSs and single nucleotide variants (SNVs) across 3Mb of the AZFc GenBank sequence (Fig. 3A). Seventeen different AZFc deletion haplotypes could be distinguished and were found to be associated with 10 different Y haplogroups. Most of these AZFc haplotypes were characterised by the lack of only a small number of SNVs *not* located adjacent to one another on the physical AZFc map based on the GenBank sequence (Fig. 3B). Only the three AZFc haplotypes (12, 16 and 17) associated with Y haplogroup N, stood out as lacking the two STSs from u3 in proximal AZFc as well as all SNVs from a large contiguous region in distal AZFc (Fig. 3B). However, the heterogeneity of other discontinuous AZFc STS/SNV deletion patterns might point to a still greater plasticity of the AZFc sequence of which the molecular base is still unknown. They might be based on inversions, insertions, or deletions of distinct AZFc amplicons as found for Y lineage N, but also mutations in restriction enzyme or in primer binding sites or multiple gene conversions between the sequence alleles in homologous amplicons might cause the same AZFc heterogeneity. Real deletions of the underlying genomic DNA fragment would only be detectable on Southern blots or by appropiate Fiber-FISH experiments as demonstrated by visualisation of the gr/gr deletions (Repping et al., 2003) and g1/g2, respectively, g1/g3 deletions (Fernandes et al., 2002; 2004). Probably novel and other methods than Southern blots and Fiber-

FISH are first required to elucidate and distinguish the entire variability of the AZFc sequence expected in different human populations.

6. Genetic redundancy in AZFc

The partial AZFc deletions in men with Y-haplogroup N do not cause infertility. If they arose by an intrachromosomal recombination event between the g1 and g3 amplicons after an inversion of the b2/b3 amplicons (Fig. 2B), the AZFc sequence of men from Y lineage N should be reduced by more than 50% (3.7 Mb versus 1.5 Mb). This would suggest that the multicopy genes in AZFc and probably also in AZFb are probably functionally redundant. Based on the knowledge of the Y-chromosomal sequence and extensive testis cDNA screening programs (Lahn and Page, 1997; Vogt et al., 1997; Kuroda-Kawaguchi et al., 2001), AZFb contains eight protein-coding genes (*CDY2, EIF1AY, HSFY, PRY, RBMY1, RPS4Y2, SMCY, XKRY*) and AZFc five protein-coding genes (*BPY2, CDY1, CSPG4LY, DAZ, GOLGA2LY*), which are all transcribed in testicular tissue (Skaletsky et al., 2003) and therefore are all candidates for some function in human spermatogenesis. Gene-specific mutations associated with a particular testicular pathology have not yet been reported for any AZFb or AZFc gene. It can therefore be assumed that only complete AZFb and AZFc deletions, i.e. including the complete gene content, cause male infertility and that smaller deletions can be functionally compensated for. This might be true for AZFc but not for AZFb deletions since partial AZFb deletions are also associated with testicular pathologies, although variable and different from that of the corresponding complete AZFb deletion (Ferlin et al., 2003)

7. Instability of AZFc deletions in sperm and gonadal dysgenesis in offspring ?

Infertile men with microscopically visible abberrations in Yq11 usually have a mosaic karyotype such as 46,XYq-/45,X0 or 46,X idicY/45,X0, with a variable number of X0 cells (Sandberg, 1985; Vogt, 1996; Vogt and Fernandes, 2003). This suggests a general instability of the human Y chromosome, especially in distal Yq11 and a subsequent loss of complete Y chromosomes in sperm or during early embryogenesis of the offspring of men with AZF microdeletions. Indeed, it has been shown that X0 cells are associated with the occurrence of AZFc microdeletions (Siffroi et al., 2000; Jaruzelska et al., 2001) and a man with an AZFc deletion and a 45,X0/46,XY mosaic karyotype has been reported with ambiguous genitalia (Papadimas et al., 2001). Screening the Y chromosome of 12 patients with Turner stigmata, who had a 45X0/46XY karyotype and sexual ambiguities also revealed a high incidence of AZFc deletions (Patsalis et al., 2002). Since a wide spectrum of phenotypes, including Turner syndrome, mixed gonadal dysgenesis, male pseudohermaphroditism, mild mental retardation, and autism was reported by an international survey of prenatally-diagnosed embryos with 45X/46XY mosaicism as well (Chang et al., 1990), these findings raise serious practical concerns for infertility clinics using sperm of men with AZFc deletion for ICSI treatment (Choi et al., 2004). Indeed, using sperm of AZFc patients in ICSI, a 100% transmission of the AZFc deletion to the male offspring has already been reported (Kent-First et al., 1996; Page et al., 1999; Oates et al., 2002), although no clinical data are available which describe genital abnormalities or other somatic defects in these ICSI-AZFc-offspring. Nevertheless, the general heterogeneity and instability of the human Y chromosome as discussed in this review suggests that AZF-microdeletions can also become "*pre-mutations*" for a subsequent complete loss of the Y chromosome in the AZF deleted patients' sperms, increasing the risk of embryonic X0 cells. This inherent heterogeneity and instability of the AZF loci on the human Y chromosome should be kept in mind when counselling men with AZF deletions for the risk of their inheritance after ICSI treatment.

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