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High deletion frequency of the complete AZFa sequence in men with Sertoli-cell-only syndrome

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We have developed a rapid screening protocol for deletion analysis of the complete AZFa sequence (i.e. 792 kb) on the Y chromosome of patients with idiopathic Sertoli-cell-only (SCO) syndrome. This Y deletion was mapped earlier in proximal Yq11 and first found in the Y chromosome of the SCO patient JOLAR, now designated as the AZFa reference patient. We now show that similar AZFa deletions occur with a frequency of 9% in the SCO patient group. In two multiplex polymerase chain reaction experiments, deletions of the complete AZFa sequence were identified by a typical deletion pattern of four new sequence-tagged sites (STS): AZFa-prox1, positive; AZFa-prox2, negative; AZFa-dist1, negative; AZFa-dist2, positive. The STS were established in the proximal and distal neighbourhoods of the two retroviral sequence blocks (HERV15yq1 and HERV15yq2) which encompass the break-point sites for AZFa deletions of the human Y chromosome. We have found deletions of the complete AZFa sequence always associated with a uniform SCO pattern on testicular biopsies. Patients with other testicular histologies as described in the literature and in this paper have only partial AZFa deletions. The current AZFa screening protocols can therefore be improved by analysing the extension of AZFa deletions. This may provide a valuable prognostic tool for infertility clinics performing testicular sperm extraction, as it would enable the exclusion of AZFa patients with a complete SCO syndrome.

Key words: AZFa deletions/AZFa STS multiplex PCR/idiopathic male infertility/human Y chromosome/SCO syndrome

Introduction

Approximately 15% of the men seeking help at infertility clinics suffer from idiopathic azoospermia, i.e. the absence of mature sperm cells (spermatozoa) in seminal fluid. In 5–20% of these patients, the molecular analysis of their Y chromosome revealed a de-novo microdeletion of at least one of the three azoospermia factor regions, AZFa, AZFb and AZFc (Vogt *et al*., 1996; Girardi *et al*., 1997; Vogt, 1998; Kleiman *et al*., 1999; Krausz *et al*., 1999). In many infertility clinics, this has led to a broad interest in the molecular analysis of Y chromosomal microdeletions in men with idiopathic azoospermia or severe oligozoospermia and numerous reports have now confirmed the presence of the three AZF loci in Yq11.

However, there is no consensus on the number of sequence-tagged site (STS) loci which have to be analysed in order to identify the detected AZF deletion as a causative agent of the observed male infertility phenotype and many infertility clinics have restricted their AZF deletion analyses to only one or two STS loci in each AZF region to reduce the time and costs involved with the extensive screening programme originally proposed (Vogt *et al*., 1996). Indeed, the detection of a deletion at only one of the non-polymorphic STS loci mapped to any of the three AZF loci would be sufficient to diagnose an AZF deletion if the molecular extension of the deletion is identical for all patients, as reported for the reference patients published by Vogt *et al*. (1996). However, deletions may have variable extents in different patients, so single STS analysis is not an adequate evaluation for deletion detection of an AZF region.

Partial AZF deletions have been identified repeatedly in different infertility clinics [e.g., for AZFa: (Ferlin *et al*., 1999); for AZFb: (Krausz *et al*., 2000); for AZFc: (Stuppia *et al*., 1997)] and were not always associated with the testicular pathologies proposed by Vogt *et al*. (1996) to occur with the AZFa, AZFb, and AZFc deletions, but were represented by variable testicular pathologies. Therefore, AZF screening programmes based on the analysis of single STS loci within an AZF region have only limited clinical value, unless the extent of the observed AZF deletion is analysed in further polymerase chain reaction (PCR) experiments.

Since the complete human proximal Yq11 region has now been sequenced (GenBank accession no. NT_011875), the sequence of a complete AZFa deletion, as defined by the molecular extension of the Y deletion of the AZFa reference patient JOLAR (Vogt *et al*., 1996), can now be estimated. Both borders of the AZFa deletion for JOLAR were recently mapped to a long identical sequence domain of two recombined retroviral sequence blocks (HERV15yq1 and HERV15yq2) present at a distance of 781.557 kb in proximal Yq11 (Kamp *et al*., 2000). Similar results have been found for eight further Sertoli-cell-only (SCO) patients with a complete AZFa deletion (Blanco *et al*., 2000; Kamp *et al*., 2000; Sun *et al*., 2000). Therefore, complete AZFa deletions can now be defined as the result of a homologous intrachromosomal recombination event between two HERV15 sequence blocks located in proximal Yq11, intervals D3 and D6 respectively.

Based on this knowledge, we developed a novel AZFa screening protocol based on a series of new STS markers (AZFa-prox1, AZFa-prox2, AZFa-dist1, AZFa-dist2) established around both HERV15 sequence blocks. Analysis of the sY83 and 12f2 STS loci can be included to map the site of the HERV15yq1/HERV15yq2 recombination event (ID1 or ID2). We combined these STS in two PCR multiplex mixes: AZFa-prox and AZFa-dist, and tested this protocol on the Y chromosomes of 100 patients with a histologically proven SCO syndrome. Complete AZFa deletions were found with a frequency of 9% in this SCO patient group.

Materials and methods

Selection of SCO patients

The 100 individuals in our SCO patient group were evaluated clinically and found to have non-obstructive (secretory) azoospermia without evidence of testicular tumours or other abnormalities. A histological diagnosis of complete germ cell aplasia (SCO syndrome) was made after careful examination of bilateral testis biopsies showed that all seminiferous tubules from both testes had no germ cells present. In this context, it is important to note that the complete absence of germ cells was diagnosed by a single biopsy from each testis. Although most of our patients were then expected to have a complete SCO syndrome throughout their testes, the presence of isolated tubuli with germ cells could not be fully excluded. The karyotype in all SCO patients was found to be normal, 46,XY.

Genomic DNA isolation from blood samples and testicular testis biopsies

Genomic DNA was extracted from blood samples (79 patients) collected in EDTA tubes by a published protocol (Vogt *et al*., 1996) and from frozen/thawed tissue samples of testicular biopsies (21 patients) collected in SpermFreeze medium (FertiPro N.V. cat. no. 3080) using the protocol described in the Ambion kit for simultaneous extraction of RNA and DNA from tissue samples (Technical Bulletin 161; Ambion, Austin, TX, USA). Briefly, 50–100 mg of testicular tissue (normal weight of biopsy samples) was placed in a volume of 500 µl denaturation solution and disrupted by an Ultra Turrax T8 tissue homogenizer using the dispersing tool S8N-5G for mini-volumes in a 2 ml plastic tube. DNA collected in the organic phases was extracted with an equal volume of extraction buffer (0.1 mol/l NaCl, 10 mmol/l Tris–HCl pH 8, 1 mmol/l EDTA, 1% SDS) after the pH value was adjusted to pH 7–8 with 5 N NaOH. The DNA samples, now in the extraction buffer, were precipitated by the addition of 1/15 vol. 7.5 mol/l ammonium acetate and 2 vol. of ice-cold ethanol. DNA pellets were rinsed with 70% ethanol and dissolved in 40 µl of TE buffer (10 mmol/l Tris–HCl, pH 8, 0.1 mmol/l EDTA) by a short heat pulse (5 min at 55°C). For each AZFa multiplex PCR experiment, a 2 µl aliqout of this DNA solution was used.

Multiplex PCR assay for complete AZFa deletions

A series of new Y-specific STS was established to analyse the proximal (AZFa-prox1 and AZFa-prox2) and the distal (AZFa-dist1 and AZFa-dist2) genomic Y regions present and absent in patients with AZFa deletions (Figure 1). AZFa-prox1 and AZFa-prox2 flank the HERV15yq1 sequence block in Yq11 interval D3 recently identified to be the proximal borderline of complete AZFa deletions (Kamp *et al*., 2000); AZFa-dist1 and AZFa-dist2 flank the HERV15yq2 sequence block in Yq11 interval D6, recently identified to be the distal borderline of complete AZFa deletions (Kamp *et al*., 2000). In the same protocol, primers for the STS sY83 and 12f2 could be added (sY83 for ID1 in AZFa-prox mix and 12f2 for ID2 in AZFa-dist mix) to identify the recombination site of the fused HERV15yq1/HERV15yq2 sequence blocks. Each primer mix also contained an STS identical to the ZFY and ZFX loci, to serve as a positive control in each screening experiment. The primer concentrations used in the two PCR multiplex mixes are as follows. AZFa-prox primer mix: 0.3 µmol/l AZFa-prox1 for/rev; 0.2 µmol/l AZFa-prox2 for/rev; 0.2 µmol/l sY83 L/R (optional for ID1 recombination site); 0.1 µmol/l ZFY/ZFX for/rev (positive control); AZFa-dist primer mix: 0.4 µmol/l AZFa-dist 1 for/rev; 0.1 µmol/l AZFa-dist2 for/rev; 0.2 µmol/l 12f2 for/rev (optional for ID2 recombination site); 0.1 µmol/l ZFY/ZFX for/rev.

All primer sequences are given in Table I and were deposited in the GenBank under the given accession numbers. Both PCR reactions were performed in a 50 µl reaction volume with 1×PCR buffer (Gibco), using 5 mmol/l $MgCl_2$ for AZFa-prox and 2.5 mmol/l $MgCl_2$ for the AZFa-dist reaction, with 0.2 mmol/l dNTP, and 2 IU Taq DNA Polymerase (Gibco) with 200 ng of the genomic patient DNA.

For lower DNA concentrations (usually when extracted from tissue samples of testis biopsies) both AZFa primer mixes could also be run without including STS sY83 and ZFX/ZFY in AZFa-prox and 12f2 in AZFa-dist, respectively, to ensure a sufficient template concentration for each PCR reaction.

Appropriate positive and negative genomic DNA controls in each multiplex PCR experiment were provided by DNA samples from a fertile man and from a woman respectively. The DNA sample from the fertile man was a control for the sensitivity and specificity of each performed PCR assay, and the female DNA sample controlled for the Y specificity of the primer pairs used and also for putative contamination. In addition, a water sample containing all reaction components with the exception of the genomic DNA sample was run with each set of primers in order to detect possible reagent contamination.

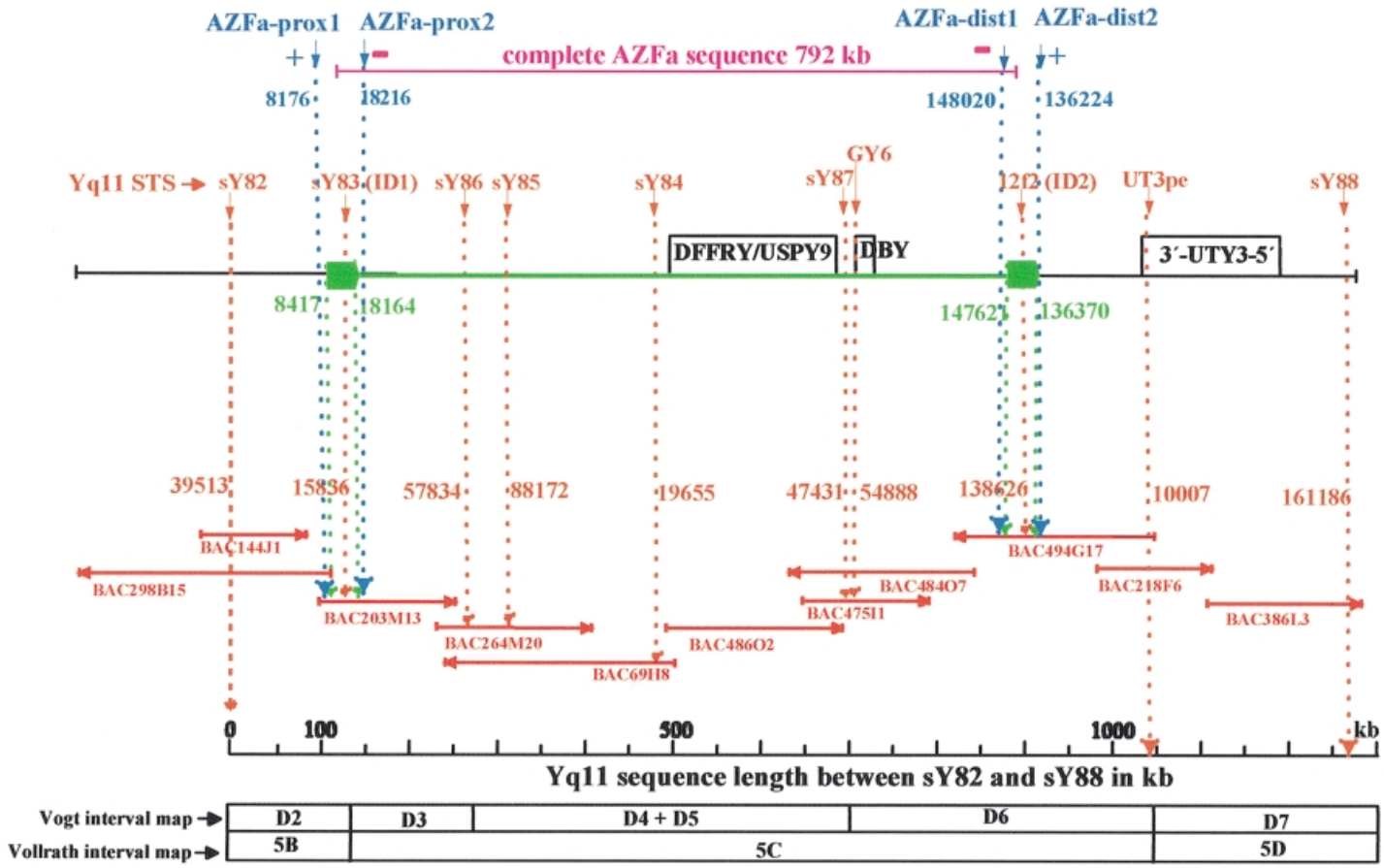

Figure 1. Schematic comparison of the proximal Yq11 sequence between the STS loci sY82 and sY88 (i.e. 1280 kb) derived from the BAC sequence contig NT_01875, published in the GenBank, and the Yq11 interval maps of Vollrath *et al*. (1992) and Vogt *et al*. (1996). Yq11 interval D4 and D5 were fused because re-analysis of the H17 patient (Vogt *et al*., 1996) revealed a deletion of sY84, sY85 and sY86 (G.Schlueter, unpublished results). The BAC sequence positions (always 5′ end of STS forward primer) of the STS: sY82, sY83, sY84, sY85, sY86, sY87, GY6, 12f2, UTY3pe and sY88 (red colour) are given in comparison to the location of the two retroviral sequence blocks, HERV15yq1 and HERV15yq2 (green colour). The location and extension of the Y genes, *DFFRY/USPY9*, *DBY* and *UTY3* are marked by appropriate black boxes. (Please note that the Y-DNA inserts in the BACs 298B15, 69H8, 484O7 and 494G17 were cloned in the BAC with reversed polarities in comparison to the genomic Yq11 centromer–telomer polarity. They are therefore displayed here with reversed polarities.) Deletion of the complete AZFa sequence (i.e. 792 kb, pink coloured) is identified by the deletion pattern of four new STS markers (blue colour): presence of AZFa-prox1 and AZFa-dist2 (+) linked to absence of AZFa-prox2 and AZFa-dist1 (–). The UT3pe STS of the UTY3 gene was established to mark the begining of Yq11, interval D7. The STS GY6 which maps in the 5′ region of the *DBY* gene was used to map the distal AZFa deletion breakpoint in the Y chromosome of patient SAYER. For further details, see text.

The PCR reaction profile (for Biometra *T*-Gradient Personal Cycler) involved a presoak for 3 min at 94°C and 33 PCR cycles, performed as follows: denaturation for 1 min at 94°C, annealing for 1 min at 63°C (AZFa-prox mix), or for 1 min at 57°C (AZFa-dist mix), with subsequent polymerization for 1 min at 65°C for both mixes and a final extension step for 2 min at 65°C. The PCR products (20 µl aliquots) were analysed on a 3% agarose gel stained with ethidium bromide. As a length marker we used the GeneRuler^{TM} 100 bp DNA ladder-plus.

Results

Mapping of complete AZFa sequence in BAC sequence contig of proximal Yq11

Originally, the AZFa region in proximal Yq11 was defined by the deletion of the Y chromosomal STS loci: sY83, sY84, sY85, sY86 and sY87 in the SCO patient JOLAR (Vogt *et al*., 1996). This corresponded to the deletion of Yq11 interval D3–D6 according to the map of Vogt *et al*. (1996), and to the deletion of Yq11 interval 5C according to the map of Vollrath *et al*. (Vollrath *et al*., 1992) respectively, and it has been suggested that the analysis of the STS loci sY82 in Yq11 interval D2 and sY88 in Yq11 interval D7 maps the AZFa borderlines (Vogt *et al*., 1996; Simoni *et al*., 1999). We have now mapped these STS loci in the BAC sequence contig in proximal Yq11 (deposited in the GenBank under the accession no. NT_011875) and compared their positions to the location of the major AZFa proximal and distal breakpoints recently found in two retroviral sequence blocks (HERV15yq1, HERV15yq2; Figure 1). The molecular length of the so-called 'AZFa sequence', i.e. the sequence deleted in reference patient JOLAR, now designated as a reference patient for complete AZFa deletions, was estimated to be 792 kb (Kamp *et al*., 2000). Selection of this reference patient to define the AZFa sequence has been confirmed since a similar AZFa deletion size was estimated in eight additional SCO patients (Blanco *et al*., 2000; Kamp *et al*., 2000; Sun *et al*., 2000).

Table I. Sequence-tagged site (STS) markers for breakpoint mapping of complete AZFa deletions

STS	Forward primer	Reverse primer	STS position in BAC of AZFa contig	PCR product length (bp)	GenBank accession no.
AZFa-prox1	AZFa-prox1-for: CTTAAATGTTGACTCTTCACC	AZFa-prox1-rev: GCCTTGTGTAGAATAAGCAGTCA	203M13: 8176–8301	126	G67788
sY83 (ID1)	sY83L: CTTGAATCAAAGAAGGCCC	sY83R: CAATTTGGTTTGGCTGACAT	203M13: 15836–16112	277	GDB:[a] 187656
AZFa-prox2	AZFa-prox2-for: GGTTCCTGAACAGGGGACT	AZFa-prox2-rev: GGCAGCAGAAGGGCCTCTC	203M13: 18216–18435	220	G67789
AZFa-dist1	AZFa-dist1-for: TTGTCCTTCAATGCAGATG	AZFa-dist1-rev: GGCTTCTAGTAGTATGGTC	494G17: 147631–148020	390	G67790
12f2 (ID2)	12f2-for: CTGACTGATCAAAATGCTTACAGATC	12f2-rev: TCTTCTAGAATTTCTTCACAGAATTG	494G17: 138199–138626	427	G67792
AZFa-dist2	AZFa-dist2-for: GCACTCCAGAAAGATAATACATC	AZFa-dist2-rev: GTTCCCCATTCATTATACTGTTAGC	494G17: 135954–136224	271	G67782
AZFa-dist3	AZFa-dist3-for CAGAGATTGCAGTGAGCAGAGACC	AZFa-dist3-rev CATGAGCCACTGAACTCAGCCTC	494G17: 133994–134993	1000	G67783
AZFa-dist4	AZFa-dist4-for GTGACCACACACTGAAAAGAAGC	AZFa-dist4-rev CAAGCATTAGTCATTAGGTGATAC	494G17: 130066–130456	391	G67784
AZFa-dist5	AZFa-dist5-for GCCTGCCAGTGCTGCTATGATAC	AZFa-dist5-rev ACTGTGTAATTTACGGTTACTTG	494G17: 102661–103167	507	G67785
AZFa-dist6	AZFa-dist6-for AGGGGCCCAAGATATATTCCTCC	AZFa-dist6-rev GGTAAAGAGTTGAAGGTTATTATG	494G17: 92115–93005	891	G67786
AZFa-dist7	AZFa-dist7-for GACTGAACATTGAACAGTGAATAC	AZFa-dist7-rev GCTGTAACACTCTTTTCTAAGTAC	494G17: 31198–31453	256	G67787
UTY3pe	UTY3pe-for AAAGTATTACGGAAGTTTGCTG	5′-UTY3pe-rev CACTGACTATATGTTTTCTACTG	494G17: 9870–10007	138	G67791

[a]STS sY83 is part of the DYS11 DNA locus and only deposited in the Genome database (www.gdb.org).

Based on this BAC sequence map between Yq11 interval D2–D7, sY82, marking Yq11 interval D2, is located at a distance of 109.692 kb proximal to HERV15yq1, and sY88, marking Yq11 interval D7, is located 369.748 kb distal to HERV15yq2 (Figure 1). Therefore, their use as STS loci for marking the AZFa borderlines requires critical revision.

Novel PCR multiplex protocol for analysis of complete AZFa deletions

With the knowledge of the complete AZFa sequence, a novel screening protocol focusing on the direct analysis of complete AZFa deletions was developed. It was based on a series of new STS markers that map around the two homologous retroviral sequence blocks HERV15yq1 (AZFa-prox1 and AZFa-prox2) and HERV15yq2 (AZFa-dist1 and AZFa-dist2) (Figure 1; Table I). The site of the HERV15yq1/HERV15yq2 recombination event (ID1 or ID2) can be analysed with the STS loci sY83 (deleted if ID1 recombination event) and 12f2 (deleted if ID2 recombination event). For further fine-mapping experiments, five novel STS (AZFa-dist3 to AZFa-dist7) were established in Yq11 interval D6, located distal to the HERV15yq2 sequence (Table I). We also established a new STS from the 3′ untranslated region (UTR) of the UTY3 (Ubiquitous TPR motif Y) (Lahn and Page, 1997) gene (UTY3pe) to mark the beginning of Yq11, interval D7. The UTY3 gene (GenBank accession no. AF000994) has a 3′–5′ polarity towards the Y centromere in Yq11.

For a convenient parallel analysis of the six AZFa STS, we established two PCR multiplex mixes. The proximal AZFa multiplex mix (AZFa-prox) contained the STS for AZFa-prox1, AZFa-prox2 and sY83, and the distal AZFa multiplex mix (AZFa-dist) contained the STS for AZFa-dist1, AZFa-dist2 and 12f2. Using the guidelines of good laboratory practice and internal quality control, and also to allow trouble-shooting in diagnostic PCR amplification techniques (Neumaier *et al*., 1998), we introduced an additional PCR analysis of the ZFY/ZFX gene as a positive control in each multiplex PCR experiment. The ZFY and ZFX PCR amplification products are identical and therefore always visible in the patients' DNA samples, even when the complete Yq11 and the proximal Yp11 region is deleted (e.g. in XX males) due to the X chromosomal location of the *ZFX* gene.

We expected the presence of STS AZFa-prox1 and AZFa-dist2 to be linked to the absence of STS AZFa-prox2 and AZFa-dist1 in the event of a deletion of the complete AZFa sequence in the patient's genomic Y DNA (see Figure 1). Additional deletion of sY83 was expected when the observed AZFa deletion was the result of a recombination event in the ID1 domain of the HERV15 sequence blocks; whereas deletion of the 12f2 locus would indicate that the observed AZFa deletion was the result of a recombination event in the ID2 domain of both HERV15 sequence blocks. Presence of both STS, sY83 and 12f2, would identify a partial AZFa deletion. Examples of the typical STS deletion patterns for both types of complete AZFa deletions are given in Figure 2.

Deletion analysis of the complete AZFa sequence in 100 men with complete SCO syndrome

We previously proposed that deletion of the complete AZFa sequence in the Y chromosome of infertile men is associated histologically with a complete SCO syndrome (Vogt *et al*., 1996; Vogt, 1998). Using our novel AZFa PCR multiplex system, we therefore analysed the presence of complete AZFa deletions in

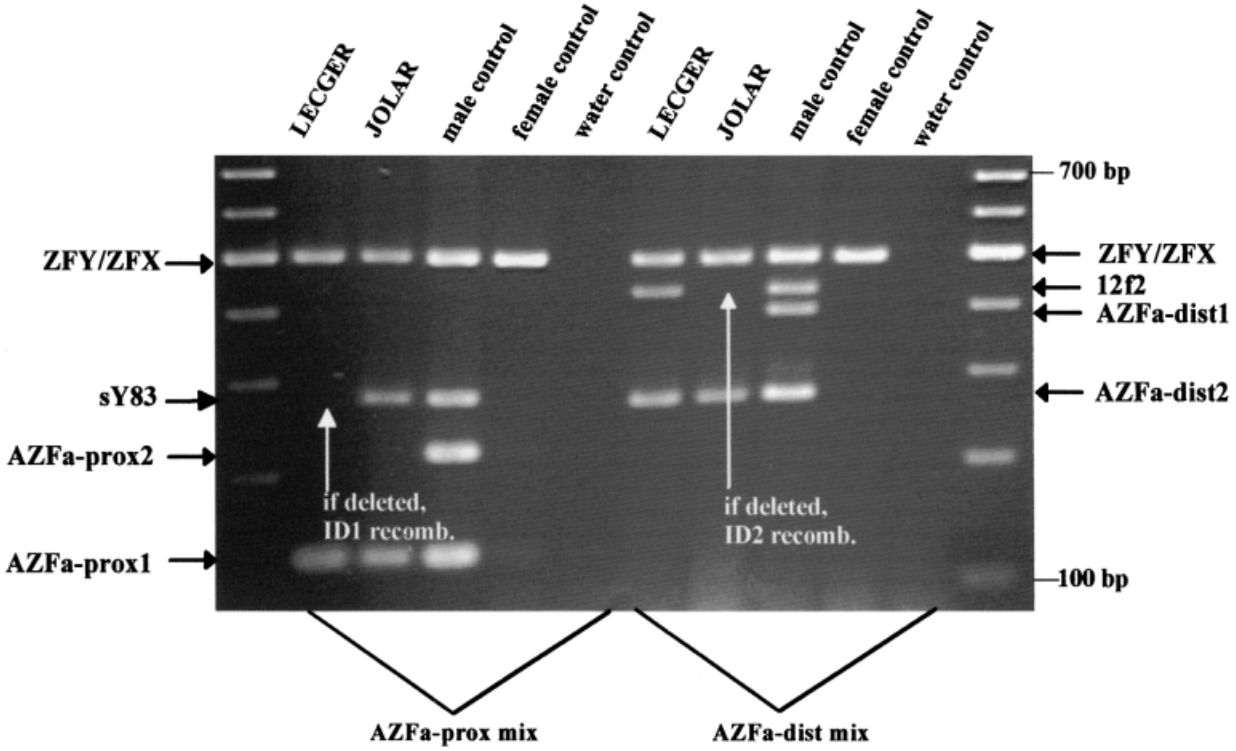

Figure 2. Two PCR multiplex experiments (AZFa-prox and AZFa-dist) identify the deletion of the complete AZFa sequence. For the locations of the used STS loci and the HERV15yq1 and HERV15yq2 sequence blocks in proximal Yq11, see Figure 1. The molecular lengths of the genomic PCR products are given in Table I. As a length marker, we used the Gibco Gene Ruler^{TM} 100 bp DNA ladder (100–700 bp) displayed at the right and the left. Identical PCR products for the *ZFY* and *ZFX* genes served as a positive control in each PCR experiment. The water sample served to control for possible contamination. DNA samples of a fertile male and female were used to control the sensitivity and Y-specificity of each PCR reaction. Deletion of the complete AZFa sequence in the SCO patients LECGER and JOLAR is shown by the presence of AZFa-prox1 and absence of AZFa-prox2 in the AZFa-prox mix, respectively, the absence of AZFa-dist1 and presence of AZF-dist2 in the AZFa-dist mix. For identification of the HERV15yq1/HERV15yq2 recombination sites (ID1 or ID2) in both patients, the sY83 STS was included in the AZFa-prox mix and the 12f2 STS was included in the AZFa-dist mix. In SCO patient LECGER, sY83 is absent, indicating recombination in ID1. In the SCO patient JOLAR, 12f2 is absent, indicating recombination in the ID2 domain. For further details, see text.

Table II. Sertoli cell-only (SCO) patients with deletions of the complete AZFa sequence

SCO patients	sY82	AZFa-prox1	AZFa-prox2	AZFa-dist1	AZFa-dist2	AZFa-dist4	sY83 (ID1)	12f2 (ID2)
ANDSCH	+	+	–	–	+	+	–	+
ERWTHO	+	+	–	–	+	+	–	+
GERKEL	+	+	–	–	+	+	–	+
HERREI	+	+	–	–	+	+	+	–
JOLAR	+	+	–	–	+	+	+	–
LECGER	+	+	–	–	+	+	–	+
LÜB-8	+	–	–	–	–	–	–	–
LÜM-17	+	–	–	–	–	–	–	–
SCO-2	+	+	–	–	–	+	–	+
STS diagnostic pattern for complete AZFa deletion		+	**–**	**–**	+	+	**if negative, ID1-rec.**	**if negative, ID2-rec.**

rec. = recombination.

100 patients with a histologically proven complete SCO syndrome.

Of the 100 SCO patients, six (i.e. 6%) showed the STS deletion pattern expected for complete AZFa sequence deletions, i.e. AZFa-prox1 and AZFa-dist2 present but AZFa-prox2 and AZFa-dist1 absent (Table II). Four of them (ANDSCH, ERWTHO, GERKEL, LECGER) had their HERV15yq1 and HERV15yq2 recombination site in the ID1 domain, shown by additional deletion of the sY83 STS. Two of them (JOLAR, HERREI) had their HERV15yq1/HERV15yq2 recombination site in the ID2 domain shown by deletion of the 12f2 STS. Since, in earlier experiments, we were able to bridge the AZFa break-fusion regions of the same six SCO patients by PCR with primers from the recombined HERV15yq1/HERV15yq2 sequence

blocks (Kamp *et al*., 2000), our novel AZFa PCR multiplex system confirmed its reliability for the direct analysis of complete AZFa deletion, and also confirmed that the mean length of a complete AZFa sequence in proximal Yq11 is 792 kb (Figure 1).

Interestingly, we found one SCO patient (SCO-2) with a complete AZFa deletion, where the distal breakpoint in Yq11 interval D6 was located beyond the HERV15yq2 sequence block. His DNA sample did not amplify the STS AZFa-dist2 marking the distal borderline of the HERV15yq2 sequence and AZFa-dist3, but was positive for AZFa-dist4 located ~5.5 kb distal to HERV15yq2. The deletion of sY83 reflected a recombination event in the ID1 domain of the HERV15yq1/HERV15yq2 sequence blocks as the apparent cause of the AZFa deletion in this SCO patient.

AZFa analysis of the SCO patients LÜB-8 and LÜM-17 indicated only the presence of sY82 in Yq11 interval D2 and the absence of all STS in interval D6 (Table II). We therefore extended the Y-DNA analysis of both patients using the UTY3pe marker for analysis of Yq11 interval D7 and using a series of markers spanning Yq11 interval D8–D25 in the multiplex PCR protocol described in Vogt *et al*. (1996). We found that all STS distal to sY82 were also absent in the Y chromosome of both patients. This was surprising because it suggests that the long arm of the Y chromosome is deleted completely in both patients, although their karyotypes were reported to be 'normal, 46,XY' (W.Küpker, personal communication). Our analysis was further confirmed when it was shown that STS sY160 (Vollrath *et al*., 1992), a marker for Y chromosome heterochromatin, was also absent on the Y chromosome of both patients. This observation reinforces the value of combining both molecular and traditional cytogenetic analyses of these patients (Rucker *et al*., 1998.)

Discussion

Complete AZFa deletions are always associated with an SCO syndrome

After having screened more than 1000 patients with idiopathic azoospermia or severe oligozoospermia in our laboratories, the specific testicular phenotype associated with each AZF deletion, as originally suggested by Vogt *et al*. (1996), has been observed only when the molecular extension of the detected AZF deletion is comparable with those originally described by Vogt *et al*. (1996). This observation reflects the fact that each AZF region contains multiple spermatogenic genes that are present within each of the AZF deletion intervals (Lahn and Page, 1997; Vogt, 1998).

In the AZFa region, a splice site mutation or complete deletions of the *Drosophila Fat Facets Related Y* (*DFFRY*) gene, also named *Ubiquitin-specific protease 9 Y* (*USP9Y*), were shown to be associated with severe hypospermatogenesis (Ferlin *et al*., 1999; Sun *et al*., 1999) and deletions of the *Dead Box Y* (*DBY*) gene were found to be associated with variable testicular pathologies including the SCO syndrome (Foresta *et al*., 2000). For partial deletions of the AZFa region, the presence of one or both Y genes may still be associated with at least focal preservation of germ cells. When both spermatogenic genes (*DFFRY*/*USP9Y* and *DBY*) are absent, a complete SCO syndrome (the proposed AZFa histopathology) has, in our experience, always been observed.

Since it cannot yet be ruled out that more Y genes are present in the AZFa region (Sargent *et al*., 1999), it is also possible that determination of complete SCO pathology is predicted by absence of the complete *AZFa* gene content (deletion of the complete AZFa sequence) now estimated at 792 kb in the AZFa reference patient JOLAR. Until this uncertainty has been resolved, it is advisable to limit prediction of a histologic diagnosis of complete SCO syndrome to those patients who have been verified to have a Y chromosome microdeletion of a molecular length comparable to that of the AZFa-reference patient JOLAR (Figure 1).

For this purpose we established the rapid AZFa screening protocol presented in this paper. It is focused on the deletion analysis of complete AZFa deletions caused by intrachromosomal recombination events of two retroviral sequence blocks in Yq 11, interval D3 (HERV15yq1) and in Yq11, interval D6 (HERV15yq2) (Blanco *et al*., 2000; Kamp *et al*., 2000; Sun *et al*., 2000) Complete AZFa deletion events were revealed by a typical STS deletion pattern in two PCR multiplex mixes: AZFa-prox and AZFa-dist (Figures 1 and 2) and the putative HERV15yq1/HERV15yq2 recombination site (ID1 or ID2) was identified by deletion analysis of sY83 (ID1) and 12f2 (ID2) respectively. We confirmed the reliability of this AZFa screening protocol by its application to 100 DNA samples from patients with a histologically proven SCO syndrome.

Nine SCO patients with a complete AZFa deletion were identified. The distal AZFa breakpoint in the SCO-2 patient was mapped 5.5 kb distal to the HERV15yq2 sequence block. This pointed to the possibility that a primary AZFa deletion event caused by recombination of the HERV15yq1 and HERV15yq2 sequence blocks could be extended by additional small secondary deletion events probably occurring stochastically in the neighbourhood of the first breakpoint fusion region.

The primary sites for the terminal Yq deletions in the Y chromosomes of LÜB-8 and LÜM-17 were difficult to identify but their proximal Yq11 breakpoints were mapped in the neighbourhood of HERV15yq1, in Yq11, interval D2. A HERV15yq1 and HERV15yq2 recombination could therefore not be excluded as the primary site for this deletion event. Both deletions included the complete AZFa sequence and therefore were expected to result in a complete SCO syndrome in the patients' testes tubules. Surprisingly, a normal 46,XY karyotype was reported for both patients, although it should have been possible to detect their large Yq deletions under the microscope. It is possible that in both patients, two Yq- chromosomes fused together during their meiotic divisions to form a dicentric iso(Yp) chromosome. In metaphase plates, iso(Yp) chromosomes have a phenotype similiar to a normal Y chromosome because the kinetochore activity of the second centromere is suppressed (Daniel, 1985). Similiar Yq^{del} fusion events are frequently found in men with large Yq11 deletions and can be easily identified by fluorescent in-situ hybridization (FISH) analysis with a centromeric DNA probe (Köhler and Vogt, 1994). For practical reasons, it was not possible to perform this FISH analysis on the chromosomes of LÜB-8 and LÜM-17.

Partial AZFa deletions are not always associated with an SCO syndrome

Partial AZFa deletions would be defined by patients with detectable sY83 and 12f2 loci as well as AZFa-dist1 or AZFa-prox2 respectively, and we assume that they are not always associated with the SCO syndrome. One example, patient SAYER (Qureshi *et al*., 1996), has been proposed to have a complete AZFa deletion. This patient had severe hypospermatogenesis with postmeiotic disruption of the spermatogenic process on histology. A DNA sample from patient SAYER (kindly provided by Prof. Howard Cooke, MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK) was therefore reanalysed in our laboratory using the PCR protocol for complete AZFa deletions as described in this paper. We found that SAYER must have a partial AZFa deletion because both AZFa-dist1 and AZFa-dist2 were present in the SAYER DNA sample (C.Kamp, unpublished results).

Other partial AZFa deletions reported include the *DFFRY/USP9Y* and *DBY* deletions (Ferlin *et al*., 1999; Foresta *et al*., 2000). As expected, most of these deletions have been associated with severe hypospermatogenesis on diagnostic biopsy but not with SCO syndrome.

High frequencies of AZFa deletions occur only in patients with SCO syndrome

In the routinely performed clinical Y deletion screening programmes on DNA samples of men with idiopathic azoospermia, AZFa deletions are only rarely found. Even after selection of patients with a small testicular volume and high levels of the follicle stimulating hormone (FSH), the frequency of AZFa deletions does not increase (C.Kamp, unpublished results). On the other hand, it has been claimed that the frequency of AZFa deletions is high in patients with a histologically proven SCO syndrome although the numbers published have been variable [50%, (Forresta *et al*., 1998); 18.2%, (Blagosklonova *et al*., 2000)]. Of 18 individuals with a bilateral SCO syndrome identified by the fine needle aspiration cytology method, nine patients with a complete deletion of the AZFa region were identified (Foresta *et al*., 1998). In 22 individuals with complete SCO syndrome, identified in conventional testicular biopsies (Blagosklonova *et al*., 2000), AZFa deletions were identified in four individuals by a PCR experiment with two nested primer pairs of the *DFFRY/USP9Y* gene. In our patient group of 100 individuals, all with a histologically proven SCO pathology, we found nine complete AZFa deletions, i.e. a frequency of 9%. A high deletion frequency of the complete AZFa sequence therefore seems to occur only in the SCO patient group. In the large group of men with non-obstructive azoospermia, SCO patients have been found with a frequency of 11–27% (Tournaye *et al*., 1996; Rucker *et al*., 1998).

Improved guidelines for analysis of complete AZFa deletions

Although the number of SCO patients with a complete AZFa deletion is still small, we would predict that only complete AZFa deletions, as defined in this paper by the typical STS deletion pattern (AZFa-prox1 and AZF-dist2 present with AZFa-prox2 and AZFa-dist1 absent), will be consistently associated with the occurrence of the severe SCO testicular pathology in the patients′ testes tubules. We therefore suggest that the current European Academy of Andrology (EAA) laboratory guidelines for analysis of complete AZFa deletions (Simoni *et al*., 1999) should be revised by replacing the STS markers sY82, sY83 and sY87, sY88, for the extension analyses of the AZFa deletion, by the four novel STS markers AZFa-prox1, AZFa-prox2 and AZFa-dist1, AZFa-dist2 as presented in this paper. The STS sY82 is located ~100 kb more proximal to the HERV15yq1 than AZFa-prox1 and the STS sY88 is located 370 kb more distal to the HERV15yq2 sequence block than AZFa-dist2. Furthermore, the absence of sY83 and sY87 does not always appear to be linked to the deletion of the complete AZFa sequence. In two patients with deletion of a complete AZFa sequence due to a HERV15yq1/HERV15yq2 recombination event in the ID2 domain, sY83 was present (Figure 2) and deletion of sY87 was also found in patients with hypospermatogenesis like SAYER (Quershi *et al*., 1996). This is explained by the fact that sY87 is located 235 kb proximal to the major distal AZFa breakpoints in the HERV15yq2 sequence block (Figure 1). Indeed, with our new AZFa screening protocol, we found that STS AZFa-dist1, and also STS GY6 marking the DBY exon 1, were present in the DNA sample of SAYER (C.Kamp, unpublished results). SAYER is therefore a patient with only a partial AZFa deletion.

The STS loci sY84 and sY86 used in the EAA minimal set for the analysis of AZFa deletions (Simoni *et al*., 1999) are always deleted in patients with complete AZFa deletions. However, both are located proximal to the location of the AZFa candidate genes *DFFRY*/*USPY9* and *DBY* (Figure 1). The possible deletion of these genes can therefore not be investigated by deletion analyses of sY84 or sY86. Therefore, it might be useful in future to replace sY84 and sY86 in the EAA minimal set by suitable STS markers for deletion analysis of the *DFFRY*/*USPY9* and *DBY* genes. This work is in progress.

The association of complete AZFa deletions with a complete SCO syndrome in all seminiferous tubules requires further AZFa screening programmes. If this is confirmed, our rapid AZFa multiplex PCR protocol might become a valuable prognostic tool for infertility clinics considering testicular sperm extraction, as it would enable them to exclude AZFa patients with a complete SCO syndrome from procedures that would be predictably unsuccessful (Anniballo *et al*., 2000).

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