LETTER TO JMG

Localisation of the Y chromosome stature gene to a 700 kb interval in close proximity to the centromere

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The sex related height difference in humans is thought to be caused mainly by two components: first, a hormonal component determined by the sex dimorphism of bioactive gonadal steroids, and, second, a genetic component attributed to a Y specific growth gene, *GCY*.[1–3] Despite extensive mapping attempts for this gene on the human Y chromosome,[4–7] its precise position remains unknown. We have recently provided evidence that inappropriate cytogenetic methodology in the characterisation of Y chromosomal terminal deletions has led to some of the difficulties in elucidating the *GCY* critical region. In order to circumvent these problems, we have decided to consider only patients presenting de novo interstitial deletions for *GCY* analysis on the Y chromosome.[8] This approach allows the assignment of *GCY* to a particular chromosomal interval without excluding the presence of X0 mosaicism and/or i(Yp) and idic(Yq11) chromosomes in patients with terminal deletions. Indeed, the direct comparison of overlapping interstitial deletions in seven adult males with normal height, one male with borderline height, and one patient with short stature resulted in the confirmation of a *GCY* critical interval between markers DYZ3 and DYS11. This region roughly encompasses 1.6-1.7 Mb of genomic DNA. To improve the resolution in the region of interest close to the centromere, we established additional new STS markers specific for this part of the chromosome using our bacterial artificial chromosome (BAC)/P1 derived artificial chromosome (PAC) contig. Molecular deletion analysis using these new Y chromosomal STSs allowed us to exclude almost all of the Y chromosomal long arm as the putative location of the *GCY* growth gene and to narrow down the critical interval to a genomic region of 700 kb.

MATERIALS AND METHODS

Selection of patients

All nine patients are sterile and have deletions of either AZFa, AZFb, or AZFc or a combination thereof. They were otherwise healthy. Patients 293, JOLAR, 28, 63, and 95 have been described clinically in detail elsewhere.[9–12] Patient Y0308

Abbreviations: BAC, bacterial artificial chromosome; PAC, P1 derived artificial chromosome; SFV, sequence family variants; STS, satellite type sequences; PAR, pseudoautosomal region

Table 1 Adult height comparison of patients and their sibs

Case	Country of origin	Height of patient (cm) and SDS	National height standard (cm)	Heights of family members (cm) and SDS
293	USA	157 (SDS −2.9) Short	176.9 (SD 6.8)	(F) 170 (M) Normal (B) Normal
Y0308	USA	165.1 (SDS −1.7) Borderline/ short	176.9 (SD 6.8)	(F) 170.1 (M) 170.1 (B) 188 (SDS +1.7) (S) 167.6 (SDS −0.4)
JOLAR	UK	168 (SDS −1.0) Normal	174.7 (SD 6.7)	(F) Normal (M) Normal (B) Normal
28	Italy	175 (SDS −0.3) Normal	176.7 (SD 6.5)	(F) Normal (M) Normal
63	Ethiopia	170 (SDS +0.3) Normal	168.0 (SD 7.4)	(F) Normal (M) Normal
95	Israel	185 (SDS +1.4) Normal	175.6 (SD 6.8)	(F) Normal (M) Normal
TM	Belgium	182 (SDS +1.3) Normal	173.5 (SD 6.7)	(F) Normal (M) Normal
1947	Germany	175 (SDS −0.8) Normal	179.9 (SD 6.4)	(F) Normal (M) Normal
1972	Germany	181 (SDS +0.2) Normal	179.9 (SD 6.4)	175 (F) 165 (M) 172 (S) (SDS +1.0)

The standard deviation score (SDS) was calculated based on the equation: SDS = (X-M)/SD, where X is a person's adult height and M and SD are the mean adult height and the 1 SD of the normal male population, respectively. (M) mother, (F) father, (S) sister, (B) brother. Patients are over 21 years of age.

Table 2 Y chromosomal STSs

STS	Left primer	Right primer	Product size (bp)
SKY1	GGACATTTGGCTGCAGAGAT	TGGCAATGCACTCTCATCAT	255
SKY2	TCAGGACAGACAGGCTGCTA	CCTGCCACTGAGCTCCTTAC	~1700
SKY3	TTCTCCCTCATCTTCCAAGC	GCTTCCATCCATTAGCAAGG	167
SKY4	CCTTTCATTCCATTCTCTTCCA	CGCACTTTATGGACTGCAA	111
SKY5*	CCCTCGTCCATTTCTTTTGA	CCTCGAATTTAATGGATTGC	202
SKY6*	TCAATGGATGCACAGTGTGGC	TCCACTGAATTCCATTGCAC	328
SKY7	GGGAGTGCAAAGGGAAAGAT	CTTTCCATGGGGTGACATTC	223
SKY8	CCATTCATTCGAGTTCATTACG	ATTGGAATGGAATCGGACAG	189
SKY9	GGCCGATGGTCAAACTGTTA	GAAACGGGCTCTGAAATTCT	531
SKY10*	ATAAGGGGCAGGTTTGTCAC	GCTACTTATTCAGTGTTTAACTGACAC	329
SKY11*	AAAGTGGGTGAAGGACATGG	TTTTTGTTTGTGGCAGGTG	469
SKY12*	TTGAGTCACTGGGGATAACTG	TATGGCCCACAATCACTTCA	216
SKY13*	GGCAGCCTAGAAAGTCTTGTTC	CCCTTGGGATTTTGTCTGTT	198

Markers with an asterisk amplify DNA fragments from more than one genomic locus (see section "Restriction analysis of PCR products for detail").

corresponds to case 1 in Pryor *et al*.[13] Patients TM, 1947, and 1972 are phenotypically normal males suffering from idiopathic infertility. Genomic DNA samples were extracted from peripheral blood leucocytes (patients 28, 63, 95, Y0308, TM, 1947, and 1972) or from lymphoblastoid cell lines (patients 293 and JOLAR). DNA isolated from peripheral blood leucocytes of normal males and females served as internal controls.

Height assessment

As all the subjects are of diverse ethnic origins, height was compared to the respective national height standards (table 1). Patients were of similar age range. When possible, special attention was given to adult height comparisons between parents and sibs. Data are summarised along with the height standard deviation score (SDS) in table 1. To calculate the SDS, mean adult height and the standard deviation were taken from the corresponding national physical growth studies. Target height and target range of patient Y0308 was calculated according to Tanner *et al*.[14]

PCR analysis

Reactions were performed in a total volume of 50 µl (75 mmol/l Tris/HCl, pH 9.0, 20 mmol/l $(NH_4)_2$ SO_4, 0.1% (w/v) Tween20, 1.5 mmol/l $MgCl_2$) containing 1.0 mmol/l of each oligonucleotide primer, 100 ng genomic DNA as template, 5 U of *Taq* DNA polymerase (Eurogentec), and each dNTP at 1 mmol/l in a thermocycler (MJ Research Inc) as follows. After an initial denaturation step of 95°C for five minutes, samples were subjected to 30 cycles consisting of 30 seconds at 94°C, 30 seconds at 60°C, and one minute at 72°C followed by a final extension step of five minutes at 72°C. Multiplex PCR was carried out as previously described[15] with minor modifications. *Alu-Alu* PCR reactions were essentially carried out as previously described.[16] Amplification products smaller than 1 kb were resolved on 3% NuSieve agarose/1% SeaKem GTG agarose (FMC) in 1 × TBE (0.089 mol/l Tris-borate/0.089 mol/l boric acid/20 mmol/l EDTA, pH 8.0). For amplification products larger than 1 kb as well as products from *Alu-Alu* PCR, 1.5% SeaKem GTG agarose gels in 1 × TBE were used for separation.

PCR primers

Y specific STSs, loci, and PCR conditions have been described previously.[17–19] Sequences of new Y chromosomal STSs are listed in table 2. Y specific STSs termed SKY were either derived from YAC, BAC, and PAC end sequences or from clone internal sequences amplified by various combinations of *Alu* primers. Primers for the markers SKY10, 11, 12, and 13 were designed to amplify fragments spanning unique restriction sites within the genomic DAZ locus (SKY10 from RP11-487K20 (AC024067), RP11-70G12 (AC006983), RP11-141N04 (AC008272), RP11-366C06 (AC015973), RP11-560I18 (AC053522), RP11-175B09 (AL359453), SKY11 and SKY12 from RP11-245K04 (AC007965), RP11-100J21 (AC017005), RP11-506M09 (AC016752), RP11-589P14 (AC025246) and SKY13 from RP11-100J21 (AC017005), RP11-589P14 (AC025246), RP11-823D08 (AC073649), RP11-251M08 (AC010682), RP11-978G18 (AC073893)) in order to detect "sequence family variants" (SFVs).

Table 3 PCR/restriction digest analysis of sequence family variants in the AZFc region

STS	Restriction enzyme	BAC clones	Fragment sizes (bp) after restriction
SKY10	*Tsp*509I	487K20	279, 50
		70G12	329
		560I18	329*
SKY11	*Nla*III	245K04	217, 154, 79, 19
		506M09	233, 221, 15
SKY12	*Mse*I	245K04	88, 57, 39, 32
		506M09	145, 39, 32
SKY13	*Cac*8I/*Tfi*I	100J21	97, 83, 23
		589P14	175, 23
		251M08	97, 50, 33, 23

*The submitted sequence of the chromosome 1 derived BAC clone RP11-560I18 (AC053522) does not show a *Tsp*509I restriction site within the genomic fragment amplified by the primer pair SKY10. Restriction analysis of fragments amplified from male and female genomic DNA, from a somatic cell hybrid line containing chromosome 1 as the only chromosome of human origin, and from the BAC RP11-560I18 also shows two fragments of ~180 bp and ~155 bp, indicating a sequence error in the complete sequence of the BAC clone.

Restriction analysis of PCR products

PCR products were resolved on agarose gels, the appropriate gel bands cut out, and the DNA isolated with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc) according to the manufacturer's protocol. Fragments amplified from SKY5 and SKY6 were digested with *Taq*I and *Bsm*I, respectively. To detect SFVs at SKY10, SKY11, SKY12, and SKY13, PCR products were digested with restriction enzymes as listed in table 3.

Sequencing of BAC/PAC/YAC end fragments

DNA from BAC/PAC clones selected for end sequencing was purified with the Nucleobond PC100 Kit (Macherey-Nagel) according to the manufacturer's instructions. End fragments

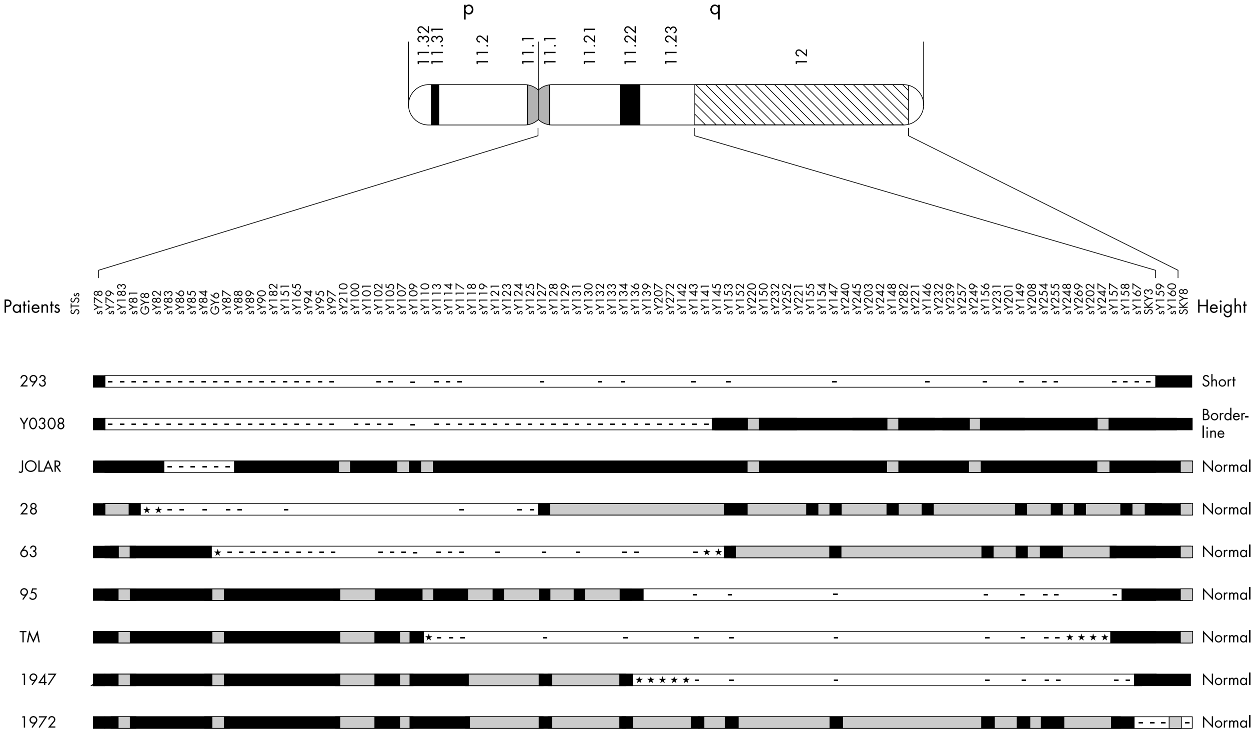


Figure 1 Deletion mapping on the long arm of the human Y chromosome. A diagram of the human Y chromosome with Yp telomere to the left and Yq telomere to the right is presented at the top. Shown below are the results of low resolution analysis of Y chromosomes of adult males with normal height or short stature. Along the top border, 95 Y chromosomal STSs are listed. Except for SKY3 and SKY8 (see table 2 for detail), all other STSs have been previously reported.[17–19] Blank spaces or grey boxes indicate inferred absence or presence of markers for which assay was not performed. Asterisks indicate markers in the respective breakpoint regions which could not be tested. In all cases where previously published data of the patients were reinvestigated, the identical DNA sample used for the primary analysis was studied. (Please note that the proximal as well as the distal breakpoint of the interstitial deletion of patient 293 resides within satellite type II sequences.)

were directly sequenced using the Thermosequenase Fluorescent Labelled Primer Cycle Sequencing Kit and analysed on a Pharmacia ALF Express (Amersham Pharmacia Biotech). YAC end fragments were generated with *Alu*/vector polymerase chain reaction and subcloned in pCR2.1 with the TOPO-TA cloning Kit (Invitrogen). Sequencing was performed as described.

Fluorescence in situ hybridisation

Metaphase spreads were obtained either from primary blood samples or immortalised cell lines. Cosmid and plasmid DNA was labelled by nick translation with biotin-16-dUTP (Roche). Slides were hybridised, stained, counterstained, and photographed according to standard protocols.[8 20]

RESULTS

Mapping of interstitial deletions

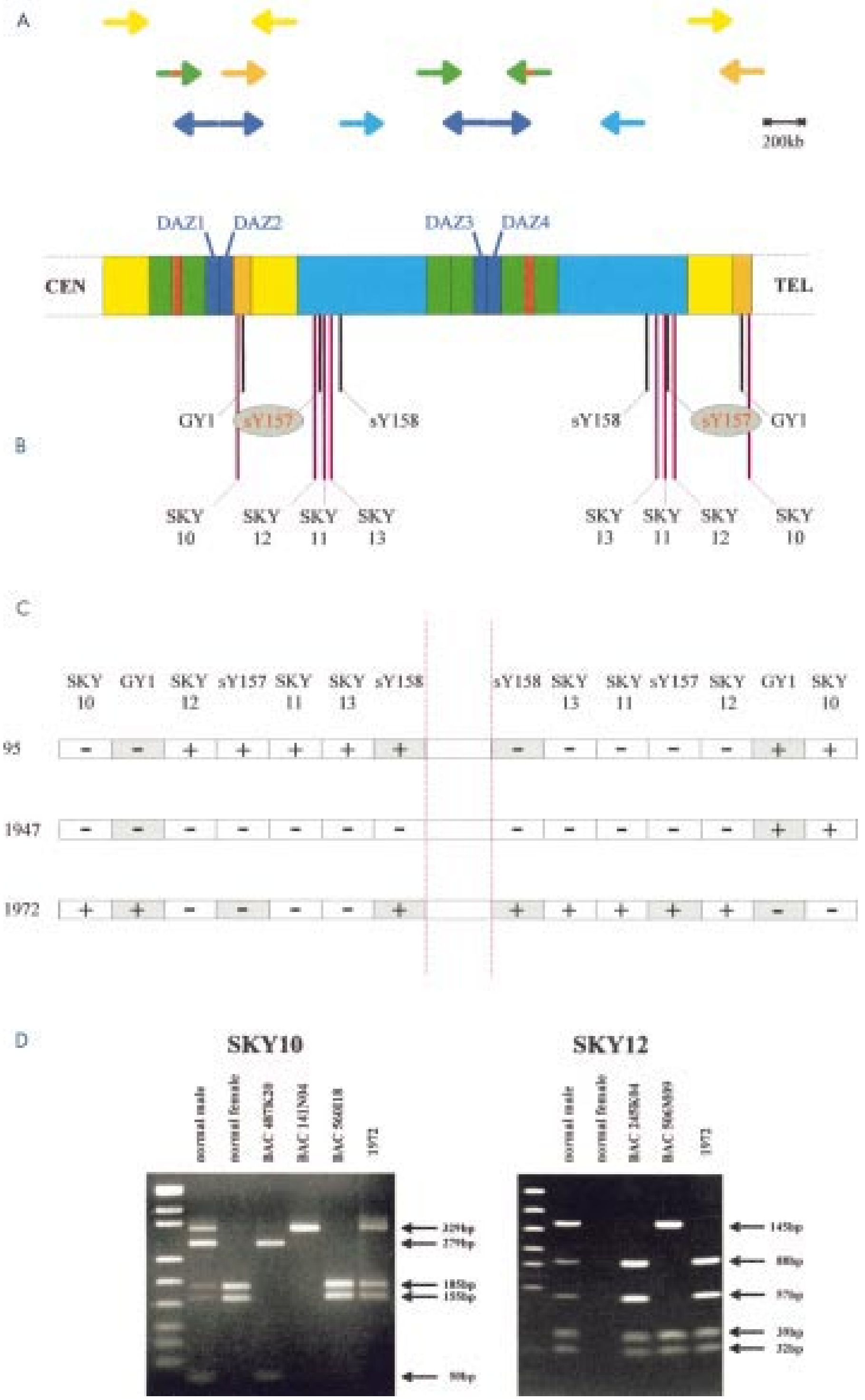
We studied the DNA of nine adult males who originally consulted reproduction centres about idiopathic infertility. Of the nine males, seven were of normal height. One patient, No 293, with a height of 157 cm, had short stature (SDS −2.9) and one, Y0308, with a height of 165.1 cm showed borderline height, being on the 3rd centile of normal US height standard (SDS −1.7). The adult height of his parents and sibs are in the normal range (table 1), his brother being 22.9 cm taller than the patient. Compared to his brother and sister on one side and to his target height (176.6 cm) and target range (166.6-186.6 cm) on the other side, he can be considered short. All men were ascertained solely on the basis of large de novo interstitial deletions on the Y chromosome. Only two of the patients had undergone previous chromosomal studies.

In our effort to localise the *GCY* locus, we focused on that part of the Y chromosome long arm that was delimited by the boundaries of the interstitial deletions of the patients with short stature (fig 1). Recently, a detailed physical map of the human Y chromosome incorporating 758 ordered STSs and 199 completely sequenced BAC clones has been constructed.[21] We used a slightly modified PCR multiplex system[15] to test the absence or presence of 28 DNA loci from the Y chromosome long arm. In patients where sufficient DNA was available for further PCR analysis, additional STSs were tested. As a result, eight of nine interstitial deletion breakpoints could be positioned (fig 1). As the deletions of patients JOLAR, 28, 63, 95, TM, and 1947, all with normal height, overlap, most of the long arm of the Y chromosome could be excluded as a critical region for *GCY*.

As the distal breakpoint of the deletion of patient 1972 does not reside within the specific part of the Y chromosome long arm, the nature of the deletion (terminal or interstitial) remained unclear. There was also no overlap of his deletion with the deletions of patients 1947 and TM. Relying solely on the results obtained by the STS based interstitial deletion mapping, the region distal to sY158 could not formally be excluded as a potential critical region for *GCY*. However, multiplex PCR analysis always showed a less intense amplification product for STS sY157 (a Y derived marker in close proximity to sY158). To address this problem, the rearranged Y chromosome of patient 1972 was investigated in more detail.

Fluorescence in situ hybridisation and sequence family variant typing of patient 1972

The overall integrity of the Y chromosome from patient 1972 was shown by FISH of cosmids LLOYNC03“M”34F05 (PAR1) and LLOYNC03“M”49B02 (PAR2) as well as the Y centromere specific probe Y-97 and the telomere specific probe “all human telomeres” (data not shown). On the basis of these results the deletion in patient 1972 was confirmed to be interstitial. Being aware of the complex structural organisation of the human DAZ



	SKY 10	GY1	SKY 12	sY157	SKY 11	SKY 13	sY158	sY158	SKY 13	SKY 11	sY157	SKY 12	GY1	SKY 10
95	−	−	+	+	+	+	+	−	−	−	−	−	+	+
1947	−	−	−	−	−	−	−	−	−	−	−	−	+	+
1972	+	+	−	−	−	−	+	+	+	+	+	+	−	−

Figure 2 Sequence family variant (SFV) typing in the human DAZ locus in distal Yq11.23. (A) Overview and amplicon structure of the human Y chromosome in the vicinity of the human DAZ cluster. Each amplicon is represented by a specific colour. Shown above are arrows indicating the orientation of each member of an amplicon family with respect to each other. The amplicon coloured orange arose from a portion of chromosome 1 that was transposed to the distal end of the DAZ cluster and partially duplicated. (B) Precise position of selected Y specific STSs and the SFVs according to the physical map of the human Y chromosome. Marker sY157 is highlighted as it was suspected to be present in only one copy by multiplex PCR analysis (see text for detail). (C) Summary of STS and SFV analysis in patients with Y chromosomal rearrangements within the human DAZ cluster region. Grey boxes indicate inferred absence or presence of markers. (D) Sequence family variant typing of SKY10 and SKY12 in genomic DNA of patient 1972. The assay is described in table 3. Along the right are listed fragment sizes (in bp). Products were separated by electrophoresis in 3% NuSieve agarose (3:1) and visualised by ethidium bromide staining.

locus (fig 2A), we specifically searched for sequence family variants (SFVs). To prevent misjudging sequence errors as single nucleotide differences, PCR/restriction digest assays were developed only from SFVs present in at least two overlapping BAC clones. The localisation of these SFVs is shown in fig 2B. As these SFVs could represent allelic variants, 10 unrelated normal German males were typed. In all cases, the expected fragment pattern could be detected for the Y chromosome derived

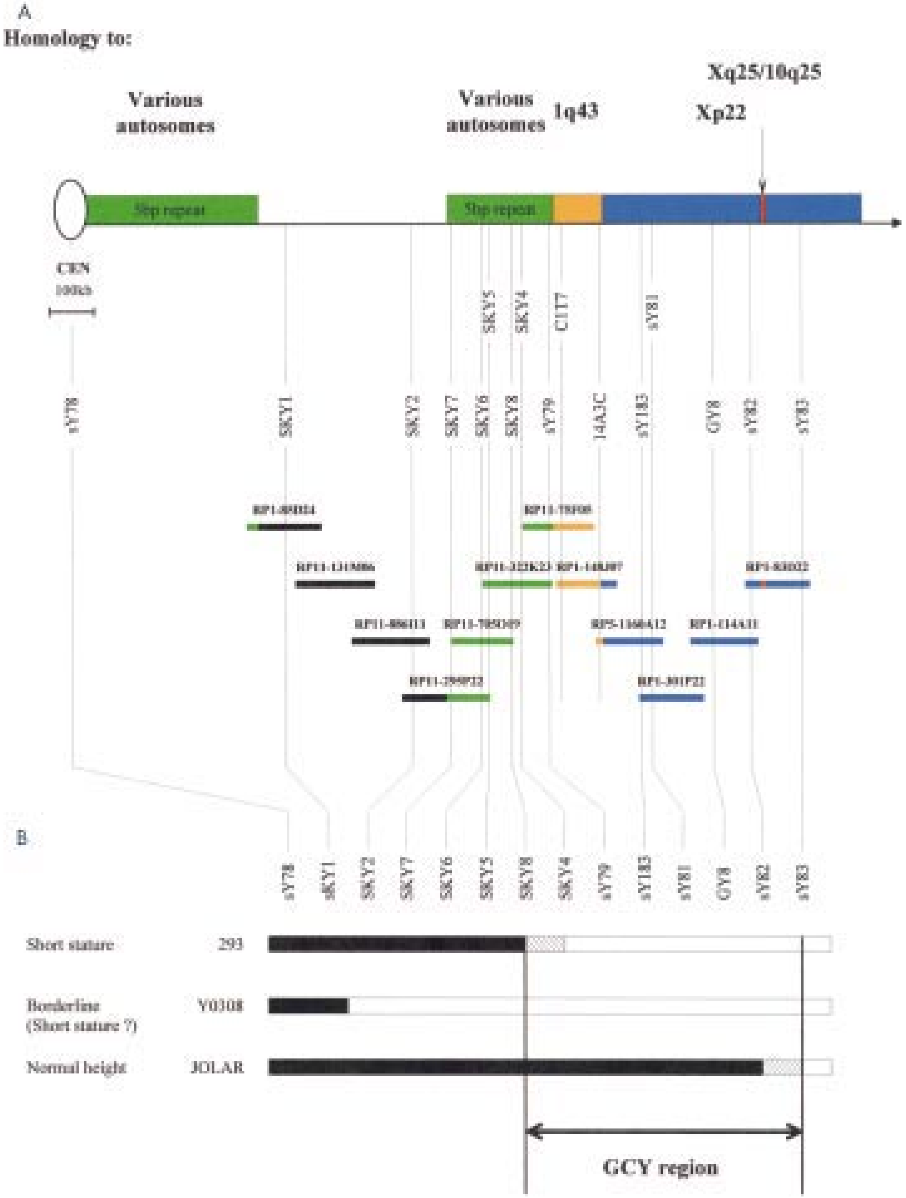


Figure 3 Schematic representation of the organisation of the long arm pericentromeric region of the human Y chromosome. (A) Diagram showing the distribution of major tandem repeat blocks and general organisation of sequence homologies. Basically, the region can be subdivided into three distinct intervals: a proximal region characterised by 5 bp satellite sequences (green), a central region with high homology to chromosome 1 (orange), and a distal region composed of X/Y homologous sequences (blue). In this X/Y homologous region, a segment homologous to chromosome Xq25/10q25 (red) has been integrated.[23 24] The precise position of the newly established and previously published STS markers in this region are illustrated below. At the bottom border, the PAC/BAC contig constructed with the aid of the new STS markers is shown. Prefixes RP1-5 indicate PAC clones and RP11 BAC clones, respectively. (B) Localisation of the *GCY* critical interval as defined by high resolution STS mapping in patients with short stature and normal height. Black boxes indicate the presence, white boxes the absence of the respective STS. Striped boxes depict the dosage unknown regions where the breakpoint resides.

sequences. In contrast, the fragment pattern deduced from the genomic sequence of the chromosome 1 derived BAC clone RP11-560I18 could not be confirmed (see table 3 for detail). Each SFV specific PCR/restriction digestion was compared to the presence/absence in the corresponding BAC clones.

Typing the genomic DNA of patient 1972 for all four sequence family variants (SKY10/*Tsp*509I, SKY11/*Nla*III, SKY12/*Mse*I, and SKY13/*Cac*8I + *Tfi*I) showed the absence of one Y derived non-allelic sequence variant (table 3 and fig 2C, D). In the case of SKY10 the distal copy is deleted. Not surprisingly, in all other typing experiments the more proximal copy of the respective SFVs was shown to be deleted.

Next, we investigated these SFVs in the two patients with the most distal breakpoints (Nos 95 and 1947). Using genomic DNA, we determined that both non-allelic variants of SKY11, SKY12, and SKY13 and one non-allelic variant of SKY10 were absent in patient 1947, whereas for all tested SFVs one non-allelic variant was absent in patient 95.

Taken together, these results provide evidence that the proximal breakpoint of the interstitial deletion present in the Y chromosome of patient 1972 resides within the interstitial deletion of patient 1947, thereby excluding this genomic region as a potential critical interval for *GCY*.

Refinement of the *GCY* critical interval

Based on the molecular analysis of the pericentric region of the long arm of the human Y chromosome,[22] the physical extension of the *GCY* critical region as defined by the markers

Table 4 Summary of BAC and PAC clones identified during physical map creation

Y STSs	Positive BACs (RPCI11)	Positive PACs (RPCI1, 3-5)
sY83	Not screened	83D22
sY82	Not screened	83D22, 114A11, 157G08, 966C15
GY8	Not screened	114A11, 168E21, 271D03, 635F21, 765H16, 806O15, 904E13, 966C15
sY81	Not screened	301P22, 1079J08, 1078C20, 1160A12
14A3C*	Not screened	148J07, 1136A14, 1160A12, 1196I23
sY79	75F05, 79E14, 102G24, 322K23, 417D23, 600D11, 612E10, 725I12, 863I08, 903M02, 1125H21	1149H11
SKY1	376B16, 544C11, 544M21	56A05, 85D24, 958M03
SKY2	79P12, 295P22, 376L20, 828O24, 886I11, 910C06	829H08
SKY4	75F05, 322K23, 612E10	Not screened
SKY5	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	Not screened
SKY6	174I24, 271E18, 295P22, 588E18, 620J20, 632F11, 684H19, 705O19	Not screened

*14A3C is a hybridisation probe previously described.[25] It detects a Y specific *Hin*dIII fragment of 3.5 kb and an additional autosomal fragment.

sY78 (DYZ3) and sY83 (DYS11) was estimated to constitute 1.6-1.7 Mb (fig 3A) of DNA. The most proximal 400 kb of this region consist exclusively of 5 bp satellite sequences separated from the Y centromere only by *Alu* sequences. This constant part of the human Y chromosome is therefore unlikely to contain coding sequences. The remainder of the *GCY* critical region is composed of X/Y homologous as well as autosomal/Y homologous sequence blocks. At the onset of this study, only limited coverage in YAC clones was available for this region. In order to refine the *GCY* critical interval and to generate gene finding substrates, it was necessary to establish a BAC/PAC contig of this region.

We generated 25 additional markers mainly by sequencing the end fragments of BAC, PAC, and YAC clones as well as clone internal sequences amplified by various combinations of *Alu-Alu* oligonucleotide primer pairs. Of these, only seven turned out to be Y specific (SKY1, SKY2, and SKY4-8) (see table 2 for detail). The BAC and PAC clones identified during the generation of the physical map are summarised in table 4. Meanwhile, some of these clones have been completely sequenced as they form part of a tiling path for sequencing the human Y chromosome.[21] The proximal part of the cloned region between markers sY78 and SKY6 has not been sequenced to date. A selection of clones covering the entire *GCY* critical region is shown in fig 3.

Confirming the overlap between BAC RP11-295P22 and BAC RP11-322K23 appeared to be the most crucial step in the process of contig construction. Y specific markers derived from the opposite end fragments of both clones were suspected to amplify identical sized fragments from two different loci within the same 5 bp satellite region. By testing several restriction enzymes known to cut frequently within 5 bp satellites composed of the consensus sequence $(TGGAA)_n$, we developed loci specific PCR/restriction digestion assays. Typing all BAC clones mapping to this sequence block with the appropriate PCR/restriction digestion assay allowed us to position them precisely, thereby confirming their overlaps.

In order to narrow down the critical interval for the *GCY* gene, we tested for the presence of the newly generated STS in patients 293, Y0308, and JOLAR. These results allowed us to define a small region for the *GCY* gene (fig 3B). Direct sequence comparison showed that the sequenced BAC clones RP11-322K23, RP11-75F05, RP11-461H06, RP11-333E09, RP11-558M10, CITB-298B15, and CITB-203M13 completely cover the mapped region between Y-STSs SKY8 and sY83 (DYS11), suggesting that it encompasses roughly 700 kb.

DISCUSSION

Since the issue of the existence of a Y specific growth gene (*GCY*) was first raised, there have been several attempts to define its precise location. Whereas initial studies unanimously pointed towards a common region on the Y chromosome long arm,[5] more recent investigations have led to the identification of two non-overlapping critical intervals.[4 6 7] FISH analyses resolved this apparent contradiction by presenting clear evidence that the patient materials used in these initial investigations contained 45,X0 cells and/or i(Yp) or idic (Yq11) chromosomes.[8] Both genetic parameters influence the adult height of a given subject, thereby rendering it impossible to predict whether such patients have lost *GCY* or not. Studies with patients carrying de novo interstitial deletions are, therefore, much better suited to address the localisation of *GCY*. In the course of reviewing published reports for patients with small interstitial deletions, in particular close to the centromere, it became clear that these patients are very rare. This prompted us to extend our search for patients carrying large de novo interstitial deletions, irrespective of their actual adult height. We examined nine adult patients, seven with normal height. Furthermore, we could show overlapping deletions, thereby excluding *GCY* from almost the entire long arm of the Y chromosome including the pseudoautosomal region 2 (PAR2). Two patients, 293 and Y0308, presented interstitial deletions enabling the restriction of the *GCY* critical region to approximately 700 kb of DNA. This region is therefore predicted to harbour one or more genes required for normal human growth.

All nine patients studied share infertility as a common phenotype, which is in agreement with their large Yq deletions. Despite extensive routine screening of infertile males in reproduction centres, only two patients were found to present borderline/short stature in combination with a confirmed large de novo deletion. We therefore conclude that cytogenetically detectable de novo deletions including the *GCY* gene are rare events. It is possible, however, that deletion sizes are generally small in this area and therefore undetectable using the presently available set of markers. In addition, the adult height reduction of 6-8 cm attributed to the Y specific growth gene[3] does not necessarily result in the diagnosis of short stature in all affected males. Sex related adult height difference is determined by the level of bioactive gonadal steroids and the Y specific growth gene. In addition, the mid-parental height strongly contributes to the final height of a person. Parameters such as nutrition, infectious diseases, and secular trend are further components influencing the adult height of a given person. Therefore, high heritability for a given trait does not prevent this trait being strongly influenced by environmental conditions.

In summary, our data suggest that *GCY* maps to a critical interval marked by the Y derived markers SKY8 and sY83 (DYS11). This 700 kb interval, recently sequenced by the Human Genome Project,[21] does not contain any known gene or any Y specific ESTs. Different reasons such as unusual gene structures, for example, genes consisting of only one exon, the lack of homology to any identified gene, or spatially or temporally restricted gene expression patterns could account for this.

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