LETTER TO JMG

Variability of sexual phenotype in 46,XX(*SRY*+) patients: the influence of spreading X inactivation versus position effects

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During male meiosis the X and Y chromosomes pair along much of their length, with a single obligatory recombination event usually occurring in the pseudo-autosomal region (PAR) at the tip of Xp and Yp,[1] thus maintaining identity of the sex chromosome PARs.[2]

Occasionally illegitimate crossover occurs outside the PAR, resulting in the transfer of Y specific sequences onto the X chromosome. Such translocations between distal Xp and Yp occur relatively frequently, resulting in the generation of 46,XX individuals, the majority of whom display an overtly male phenotype due to transfer of the *SRY* gene onto the short arm of the paternal X.[3–5] However, a small number of Yp translocations are associated with hermaphroditism, defined as the presence of both testicular and ovarian tissue in the same individual.[6–8] While the size of the translocated Yp fragment in XX males is variable,[9] a recombination hotspot defined by the X/Y homologous genes *PRKX* and *PRKY* accounts for approximately one third of cases.[10 11] A common inversion polymorphism in proximal Yp flanks this recombination hotspot.[12–14]

In XX individuals one of the two X chromosomes is inactivated in early embryonic development as a mechanism of dosage compensation for sex linked genes.[15] This results in the conversion of the inactive X to a heterochromatic state and the transcriptional silencing of most of the genes upon it.[16] Studies of X;autosome translocations have demonstrated that the X inactivation signal is also capable of spreading into *cis* linked chromatin in a variable manner,[17–19] and it has been proposed that spreading of X inactivation into the translocated Yp segment carrying the *SRY* gene could account for the incomplete masculinisation which is occasionally observed in individuals with X/Y translocations.[3 7 8] While evidence to support this view comes from studies of the *Sxr* mouse,[20 21] only a single murine study has examined the spreading of X inactivation into Y chromatin,[22] and no such studies have been performed in humans.

It is also becoming clear that chromosomal rearrangements can in themselves result in a disruption of normal gene expression, a phenomenon known as ''position effect''.[23] Studies of an increasing number of human diseases, including aniridia,[24] campomelic dysplasia,[25] and X linked deafness,[26] have shown that rearrangements, some located up to 900 kb from the affected gene, can result in transcriptional repression, perhaps by removing essential regulatory elements or by alterations of local chromatin structure.

Here we describe detailed studies of 15 individuals with segments of Yp translocated onto the distal short arm of the X chromosome. In each case we have mapped X and Y breakpoints, determined X inactivation ratios, analysed the expression and methylation status of translocated Y chromosome genes, and performed phenotype/genotype correlations. Our analysis finds little evidence for spreading of X inactivation into Yp chromatin, and instead suggests that position effects resulting from chromosomal rearrangement account for the occurrence of incomplete masculinisation in some individuals with X/Y translocation.

Key points

- We have studied causes of incomplete masculinisation in 15 individuals with segments of Yp translocated onto distal Xp. In each case we mapped X and Y breakpoints, determined X inactivation ratios, and performed expression studies of translocated Y genes by allele specific RT-PCR. We confirm the presence of a Yp inversion polymorphism predisposing to X/Y translocation.
- Expression studies found very little evidence for spreading of X inactivation into Yp chromatin. However, in several cases disruption of gene expression occurred independently of X inactivation, suggesting ''position effects'' resulting from chromosomal rearrangement.
- In particular, five of the six translocation carriers studied with an intersex phenotype showed either translocation breakpoints very close to *SRY*, or disrupted expression of genes near *SRY* in a manner unrelated to X inactivation. Furthermore, Southern analysis suggested the presence of a cryptic rearrangement 3–8 kb proximal to *SRY* in one such case.
- Overall, our observations suggest that incomplete masculinisation in cases of X/Y translocation is a result of disruption of normal *SRY* expression by position effect rather than X inactivation.

METHODS

Subjects

The 15 patients included in this study were ascertained from a number of sources, and several have been described previously. Cases 1–4 and 7 are PG, AK, RZ, JW, and KM, respectively, in Kusz *et al*[8]; further analyses detailing the familial nature of the translocations in cases 1 and 2 are also described in Sharp *et al*[27]; case 5 is described in Coles *et al*[28]; and case 15 is patient 3 in Lindsay *et al*.[29] With the exception of case 14 (a hermaphrodite), all the remaining patients presented with an overtly male phenotype or Klinefelter syndrome. Thus, patient referrals ranged from classic ''XX male'' to true hermaphroditism with ambiguous genitalia (fig 1).

Abbreviations: PAR, pseudo-autosomal region

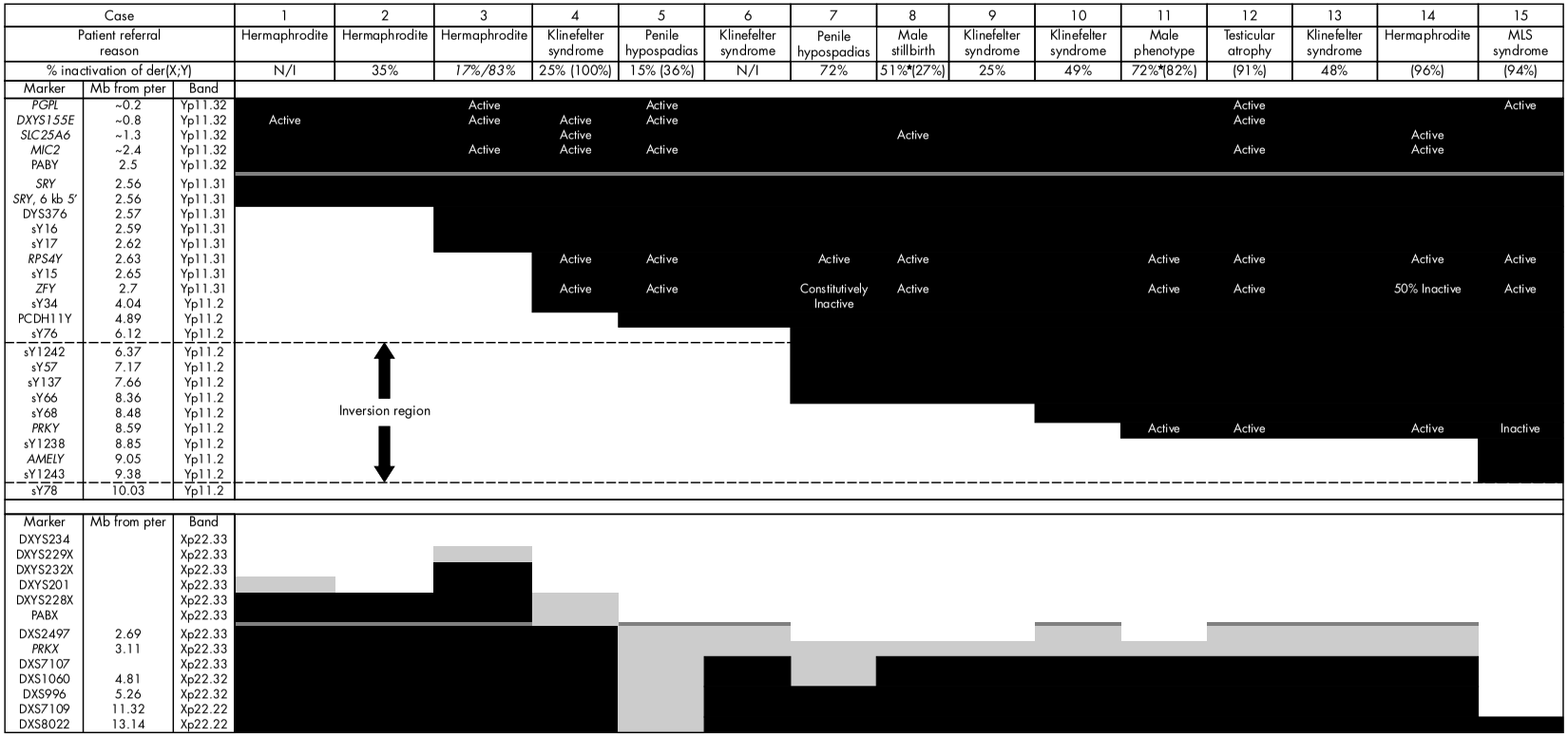

Figure 1 Clinical details, X inactivation ratios, breakpoints, and expression analysis of 15 Yp translocations. The upper section of the figure shows the translocated Yp segment, while the lower section shows the Xp breakpoints. Markers present on the der(X;Y) are shaded black, those shown in grey are non-informative, and deleted markers are unshaded. Results of RT-PCR analyses show expression status of genes in cells in which the der(X;Y) is inactive. The boundaries of the Xp/Yp PAR (PABX/PABY) are represented by grey lines. Boundaries of the Yp inversion polymorphism are represented by dashed lines; markers within this region are shown in the inverted order. In case 15 the orientation of the inversion segment is unknown, but for clarity markers are shown in the same order as in cases 7–14. In case 5 the presence of a complex (X;Y) and (X;4) rearrangement precluded mapping of the Xp breakpoint proximal to the PAR. The majority of X inactivation ratios were determined using samples of peripheral blood, except in cases 8 and 11 (asterisked) where skin and testicular fibroblasts were studied, respectively. X inactivation ratios shown in parentheses are those determined in cultured lymphoblast or fibroblast cell lines which often differ from those in vivo, N/I=non-informative. In case 3 parental origin could not be assigned and thus the X inactivation ratio is ambiguous (italicised). Mapping data were obtained from Human Genome Browser Gateway, April 2003 assembly (http://genome.cse.ucsc.edu/), except for DXS7107 and markers and genes within the PAR which were taken from Thomas *et al*[44] and Gianfrancesco *et al.*[45] Additional available clinical data for cases 1–8, 14, and 15 follows. Case 1: ambiguous internal and external genitalia, a left ovotestis with signs of dysgenesis in the testicular part, a right ovary, testosterone levels <0.1 ng/ml. Case 2: ambiguous internal and external genitalia, a left ovary, and right testis with no signs of spermatogenesis. Case 3: ambiguous internal and external genitalia, a left ovotestis with no signs of spermatogenesis in the testicular part, a right ovary, and testosterone levels of 0.6 ng/ml. Case 4: male internal and external genitalia, bilateral testes with no signs of spermatogenesis, and testosterone levels of 3.9 ng/ml. Case 5: multiple dysmorphisms consistent with Wolf-Hirschorn syndrome with a small penis, hypospadias, and small scrotum with palpable gonads. Case 6: raised FSH, LH, and reduced testosterone levels. Case 7: male genitalia, penile hypospadias, bilateral testes with apparently normal tissue adequate for age, and testosterone levels of 1 ng/ml. Case 8: a term intra-uterine death with an apparently normal male phenotype. Case 14: Ambiguous external genitalia showing micropenis with significant chordee, bifid scrotum, and penoscrotal hypospadias. Testes descended bilaterally with evidence of spermatogenesis on tissue biopsy. No evidence of mullerian structures on pelvic ultrasound. Case 15: Bilateral microphthalmia, linear skin lesions, and short stature.

Cell culture

Fibroblast and EBV transformed lymphoblastoid cell lines were established (cases 4 and 14) or obtained from the European Collection of Animal Cell Cultures, Porton Down, Wiltshire, UK (case 5, DD0144; case 8, DD3000; case 11, DD0053; case 12, DD0249; case 15, AL0049) and grown according to standard protocols. In all other cases samples of peripheral blood were obtained.

Molecular analysis

The translocated region of Y was determined in each case by the presence or absence of STS markers by PCR (fig 1). Parallel amplification of male and female control DNA confirmed Y chromosome specificity for each marker. X chromosome breakpoints were mapped by PCR amplification of polymorphic microsatellite markers (fig 1) in each proband and their parents. Breakpoints were assigned between the most proximal informative deleted marker and the most distal informative non-deleted marker. Where no parental DNA samples were available (cases 3, 6, 10, and 12–15) breakpoints were assigned distal to the most proximal heterozygous marker.

X inactivation ratios were determined by duplicate analysis using the *AR* gene PCR assay as described previously.[30] This assay has shown to be both highly reproducible[30] and quantitative over a wide dynamic range (A Sharp, unpublished data). Where this analysis was not informative, X inactivation ratios were assessed cytogenetically by combined detection of late replicating DNA and FISH using the probe pHuR14, specific to *SRY*.[18] For each case, at least 50 cells were scored for late replication, corresponding to inactivation, of the normal X or the der(X;Y).

Allele specific semi-quantitative RT-PCR of PAR and X/Y homologous genes was performed as described previously.[18] This method utilises transcribed sequence divergences or polymorphisms between X/Y homologous genes to differentiate the X and Y derived transcripts, allowing their relative levels of transcription to be quantified. For each X/Y homologous gene, pairwise BLAST alignment (http://www.ncbi.nlm.nih.gov/blast/) of mRNA reference sequences (http://www.ncbi.nlm.nih.gov/Web/Genbank) identified sequence divergences between the X and Y homologues. Webcutter 2.0 (http://www.firstmarket.com/firstmarket/cutter/cut2.html) was used to identify divergences which altered a restriction site, and spanning primers designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All primers were located in regions of perfect X/Y identity to ensure similar amplification efficiency from the X and Y homologues. For genes located within the Yp PAR, putative transcribed single-nucleotide polymorphisms were identified from LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/) and screened by PCR, restriction digestion, and agarose gel electrophoresis for heterozygosity in each case. Details of each expression assay are shown in table 1.

For allele specific RT-PCR, RNA was extracted from peripheral blood, lymphoblast, or fibroblast cell lines using TRIZOL (Gibco BRL, Paisley, UK), DNAse treated (Promega, Southampton, UK), and cDNA synthesised using M-MLV RTase (Gibco BRL) with gene specific reverse-strand primers. Two rounds of PCR were performed using unlabelled and labelled primers, respectively, to avoid heteroduplex formation,[31] which were digested and the products resolved using an ABI 377 sequencer for quantification of alleles by peak height. A gene was scored as inactive when there was a reduction in intensity of the Y specific allele, with the allele ratio gained using cDNA of the proband significantly different from that obtained using control cDNA (fig 2). Such results indicate that the copy on the der(X;Y) is inactive (fig 2). Similarly, a gene was scored as active when results obtained using cDNA of the proband were similar to those gained using control cDNA with no reduction in intensity of the Y specific allele.

Methylation analysis of CpG islands located at the 5′ end of *SRY*, *ZFY*, and *PRKY* was performed by restriction enzyme digestion with *Hpa*II, *Cfo*I, or *Msp*I and subsequent PCR, as described previously.[19]

Probes for Southern blot analysis were generated by Expand Long Range PCR (Roche, Lewes, UK) using the following primer sequences: *SRY1* for cgttccccggtactactgaa, *SRY1* rev catcttgaaaggacatttgactc (4–6 kb 5′ of *SRY*); *SRY2* for gcaggtcaccaaagacacaa, *SRY2* rev tgaatcacattttcactggga (2–4 kb 5′ of *SRY*); *ZFY* for attgttgcttagtttgcatcccaga, *ZFY* rev taccaaatatgcacaggtttgaggg (spanning exon 2 of *ZFY*); *RPS4Y* for gagaggaactgggaaaaggtgtca, *RPS4Y* rev ggatcttgccttc tacaagctcca (30–32 kb 3′ of *RPS4Y*).

To determine the orientation of a polymorphic inversion sequence within Yp we used the STS markers sY1242 and sY1243, which are specific to repeat sequences flanking the inversion (J Lange, H Skaletsky, and D Page, personal communication). Because the Yp breakpoint in cases 7–14 lay within the inversion region, its orientation could be assigned based on the presence of either one of the flanking repeats. Primer sequences are as follows: sY1242 for cgtcggtattttacgacacg, sY1242 rev gcatttgtttttcatgtgcg; sY1243 for atctgcacacttgggtaggc, sY1243 rev gaggaaatgcagaatttggg.

RESULTS

Clinical details

Although each of the 15 Yp translocations included *SRY*, as confirmed by positive PCR amplification using primers spanning the coding region of the gene, sexual phenotype varied. Patient referral reason and more detailed clinical data, where available, are shown in fig 1. While the majority of individuals studied presented with a clinical diagnosis of Klinefelter syndrome, four individuals (cases 1, 2, 3, and 14) were hermaphrodites, presenting with both male and female gonadal tissue and ambiguous genitalia, and two others (cases 5 and 7) had penile hypospadias. One individual (case 15) presented with MLS syndrome, resulting from functional nullisomy for the *HCCS* gene in Xp22.31.[32]

Table 1 Assay details used for expression studies

Gene(s)	Primer sequences and labels	Restriction enzyme	Product sizes in bp (Y allele underlined)
PGPL	A: aatggtcgtgtccaccaaaac TET, B: ggcttccagttctttcttggtc	*Aat*II	158>158+95
DXYS155E	A: ttcagtttcaaacacgtagtcctt FAM, B: cgccaagcagacacagaaat	*Nla*III	155>155+130
SLC25A6	A: agcacgccagcaagcagat, B: acagcacgccctgctcctt TET	*Bsl*I	91>91+61
MIC2	A: tcaggtggagaaggaaaagg, B: tgaagcatagcttctttttctgg FAM	*Hae*III	168>168+103
RPS4X/Y	A: ccctgctggattcatggatgt FAM, B: ctcactttgcacaacttgtactt	*Hae*III	135>110+75
ZFX/Y	A: catgatagtgtagtggaagcagaaa TET, B: atcttctccagggtcagcttta	*Bam*HI	318>249+117
PRKX/Y	A: gtctgtcctgaaggaagtcagc HEX, B: tccaagtcctgtctaccagctt	*Pst*I	310>310+170

Breakpoint analysis

There was considerable variation in the size of the translocated Yp segment in the cases analysed (fig 1). While in one patient (case 15) almost the entire short arm of the Y chromosome was translocated onto distal Xp, in other individuals (cases 1 and 2) the Yp breakpoint occurred less than 20 kb proximal to the pseudoautosomal boundary. In two of the hermaphrodites the Y breakpoint occurred 6–7 kb proximal to *SRY*, and 63–70 kb proximal to *SRY* in a third case. However, there was clearly a hotspot for illegitimate X/Y recombination close to *PRKX* and *PRKY*, consistent with previous observations.[10 11] In cases 7–14 the Yp breakpoints clustered within a region of <500 kb around *PRKY*, and the corresponding Xp breakpoints all occurred close to *PRKX* in Xp22.33. Similarly, where the Yp breakpoints were very distal (cases 1–3), Xp breakpoints occurred within the PAR, resulting in a der(X;Y) chromosome carrying two copies of the proximal part of the PAR.[4]

X inactivation ratios

There was considerable variation in the proportion of cells in which the der(X;Y) was inactive between different cases (fig 1). In the majority of cases, X inactivation ratios were determined using samples of peripheral blood. However, for several patients the only samples available were immortalised cell lines. These can show very different X inactivation ratios from those in vivo, as observed in cases 4, 5, and 8, where both fresh tissue and cultured cell lines were available and gave discordant results.

Expression analysis of translocated Y genes

Results of expression analysis of four PAR genes (*PGPL*, *DXYS155E*, *SLC25A6*, and *MIC2*) and three X/Y homologous genes (*RPS4Y*, *ZFY*, and *PRKY*) are shown in fig 1. *SRY* was not analysed because of its tissue limited expression. In addition, no RNA was available for cases 2, 6, 9, 10, and 13. Except in

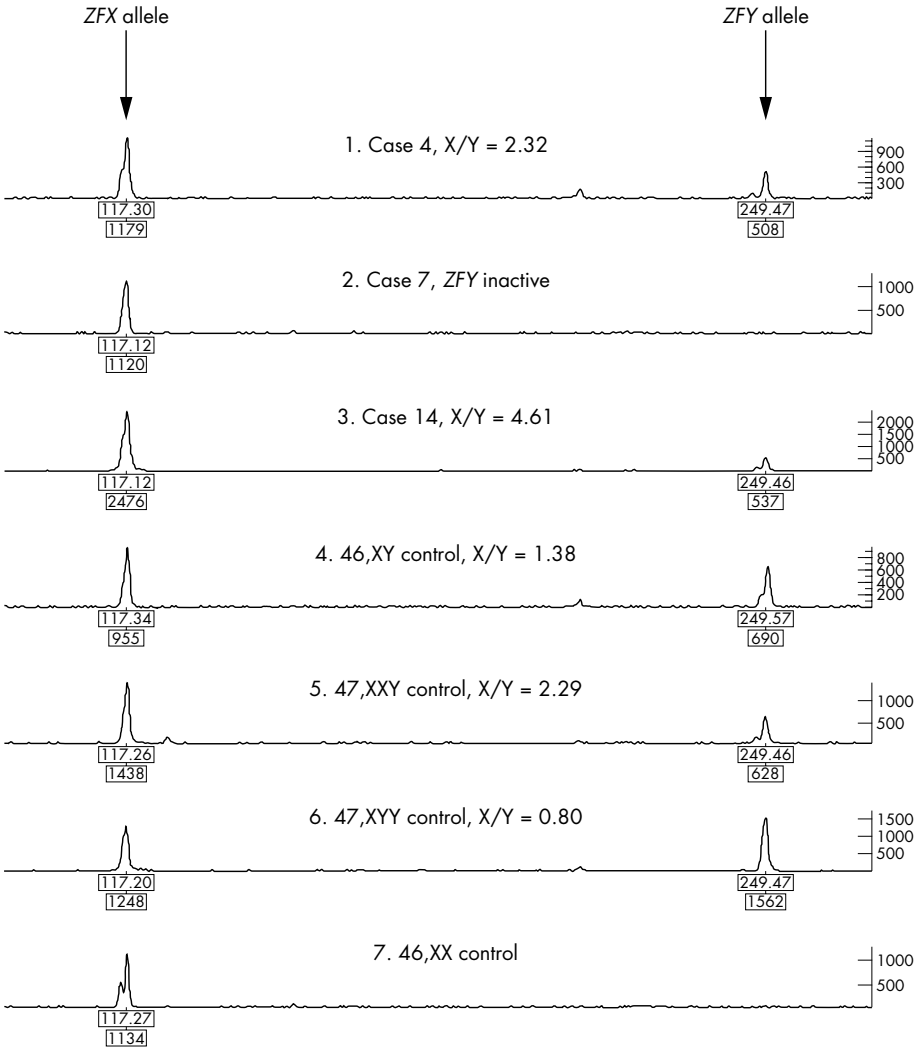

Figure 2 Results of allele specific RT-PCR of *ZFX/Y*. Following PCR of cDNA using a single primer set which amplifies both *ZFX* and *ZFY* transcripts, digestion with *Bam*HI yields a 249 bp *ZFY* specific fragment and a 117 bp *ZFX* specific fragment. The ratio "X/Y" represents the relative intensity of *ZFX* to *ZFY* by peak height in each case, and allows assessment of the relative transcription of each gene. Cases 4, 7, and 14 (tracks 1–3, respectively) each carry two copies of *ZFX* and one copy of *ZFY*. The ratio of *ZFX/ZFY* in case 4 is almost identical to that in a 47,XXY control (track 5), suggesting normal transcription of *ZFY*. However, in case 7 (track 2) *ZFY* expression is completely abolished, while in case 14 (track 3) *ZFY* expression is reduced to approximately half normal levels. Analysis of cDNA from control individuals with 46,XY, 47,XXY, and 47,XYY karyotypes (tracks 4–6, respectively) demonstrates the sensitivity of the assay to relative changes in template concentration. Figures below each allele represent size in base pairs and peak height, respectively. Each individual was tested in triplicate, with replicates yielding similar results.

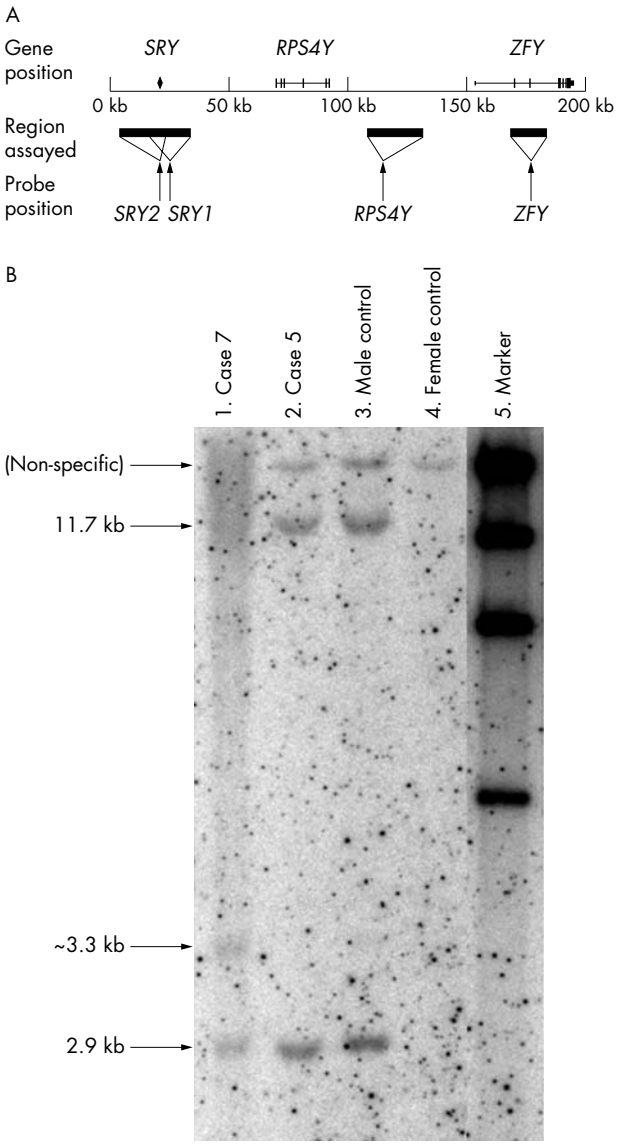

Figure 3 (A) Schematic of Yp11.31, showing genes, physical distance, and the relative locations of the four probes used for Southern analysis (arrows) and the corresponding regions investigated for cryptic rearrangements (bars). (B) Results of Southern analysis using probe *SRY1* hybridised to *Nde*I digested DNA. Case 5 (track 2) shows a hybridisation pattern identical to the male control (track 3), with Y specific *Nde*I fragments of 2.9 and 11.7 kb. However, case 7 (track 1) shows an abnormal hybridisation pattern, with a novel fragment of ~3.3 kb replacing the expected 11.7 kb fragment, suggesting the presence of a cryptic rearrangement ~3–8 kb proximal to *SRY*.

three patients (cases 7, 14, and 15) all Yp genes assayed were normally expressed.

In case 15 *PRKY* expression was reduced to <10% of normal levels. As X inactivation ratio analysis showed the der(X;Y) to be inactive in 94% of cells assayed, this suggested silencing of *PRKY* on the der(X;Y). Furthermore, analysis of a CpG island located at the 5′ end of *PRKY* using methylation sensitive restriction enzymes[19] demonstrated the presence of high levels of methylation in case 15 which were not detected either in cases 11–14 or control individuals (fig 3), indicating that silencing of *PRKY* resulted from a spreading of X inactivation into the translocated Yp segment. However, *PRKY* expression was normal in cases 11, 12, and 14.

ZFY expression was disrupted in two individuals (shown in fig 2), although in neither case did this appear to be related to X inactivation. No *ZFY* transcripts were detectable in case 7, despite the der(X;Y) being active in a proportion of cells, and *ZFY* was expressed at approximately 50% of normal levels in case 14, with an X inactivation ratio of 96:4. Additionally, analysis of a CpG island at the 5′ end of *ZFY* did not detect any methylation differences between these two patients and 46,XY males or other X/Y translocation carriers in whom *ZFY* expression was normal (data not shown).

Southern blot analysis of Yp11.31

Because cases 7 and 14 presented with both reduced *ZFY* expression, which was apparently not due to spreading of X inactivation, and also intersex phenotype indicative of abnormal *SRY* expression, we hypothesised the presence of additional cryptic rearrangements within the translocated Yp11.31 region which disrupt both *ZFY* and *SRY* expression in these individuals. Consistent with this hypothesis, Southern analysis using a probe specific to the 5′ region of *SRY* hybridised to a novel *Nde*I fragment in case 7 which was not observed in other X;Y translocation carriers or normal controls (fig 4), suggesting the possible presence of a cryptic rearrangement 3–8 kb proximal to *SRY*. Hybridisation patterns in all other cases were normal.

Analysis of a Yp inversion polymorphism

There is an inversion polymorphism of ~4 Mb in proximal Yp (fig 1) flanked by IR3 inverted repeats.[13 14] Using STS markers specific to these flanking repeats (J Lange, H Skaletsky, and D Page, personal communication) we determined the orientation of the inversion in those patients in whom the Yp breakpoint lay within the inverted region (cases 7–14). All eight individuals studied carried the inversion. As previous haplotype analysis indicates the inversion is present on approximately one third of European Y chromosomes (27/81 normal males),[12] its prevalence in Xp/Yp translocations is significantly increased ($p = 0.0003$, Fisher's exact test), confirming the suggestion of Jobling *et al*[12] that the inversion predisposes to Xp/Yp translocation.

DISCUSSION

Because X/Y translocations move *SRY* onto the X chromosome, spreading of X inactivation provides an attractive hypothesis to explain the variable sexual phenotype associated with Xp/Yp translocations.[3 7 8] While studies of the *Sxr* mouse support this hypothesis,[20 21] evidence from the study of human X/Y translocations is limited.

Our analysis allowed this hypothesis to be examined in more detail. If correct, we expected two criteria to be fulfilled: (i) patients with incomplete masculinisation would exhibit preferential silencing of the der(X;Y) when compared to classical XX males, and (ii) expression studies of translocated Yp genes would reveal evidence for spreading of X inactivation into Y chromatin. However, our data gave little support for either of these predictions.

We observed no apparent relationship between sexual phenotype and X inactivation ratios in the samples available for analysis. In particular, we did not find preferential silencing of the der(X;Y) in patients with incomplete masculinisation, as might be predicted from studies of the *Sxr* mouse.[20 21] It should be noted, however, that in most cases the only tissue available for X inactivation analysis was peripheral blood, and it is possible that this may not be representative of other tissues.[30]

Secondly, our expression studies found very little evidence for spreading of X inactivation into Yp. Unfortunately direct study of *SRY* was not possible due to its tissue limited expression, and efforts to utilise CpG island methylation as a measure of the spread of X inactivation were inconclusive because of the apparent presence of *SRY* methylation in

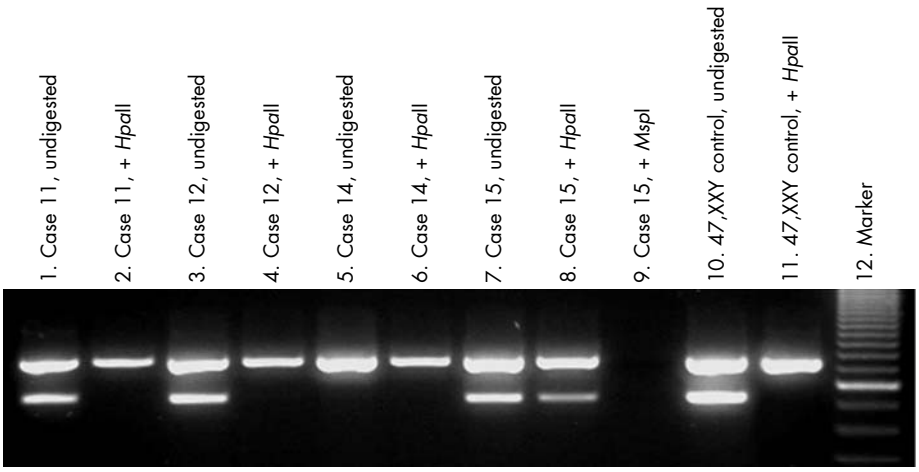

Figure 4 Results of methylation analysis of the CpG island of *PRKY*. Genomic DNA was either undigested, digested with the methylation sensitive restriction enzyme *Hpa*II, or with its methylation insensitive isoschizomer *Msp*I. Digests were then coamplified using primers spanning the CpG island of *PRKY* (lower band) and control primers spanning the CpG island of *PGK1* (upper band), an X linked gene which is known to be methylated on the inactive X and unmethylated on the active X.[43] Following digestion with *Hpa*II only methylated DNA remains intact and available as template in the subsequent PCR reaction. Analysis of control DNA from a 47,XXY individual (tracks 10 and 11) shows the CpG island of *PRKY* to be normally unmethylated, represented by its failure to amplify following *Hpa*II digestion. Analysis of cases 11 and 12 (tracks 1–4) similarly shows that the *PRKY* CpG island is unmethylated in these individuals, consistent with results gained by RT-PCR which showed normal *PRKY* transcription in these cases (fig 4). However, in case 15 PCR of DNA which has been digested with *Hpa*II still amplifies the CpG island of *PRKY* (track 8), but does not following digestion with *Msp*I (track 9), demonstrating the presence of *PRKY* methylation in this individual. This result is consistent with those gained by RT-PCR of *PRKY*, and suggests silencing of *PRKY* by a spreading of X inactivation. In case 14 even using undigested DNA no *PRKY* amplification was observed (track 5), suggesting that the Yp breakpoint in this case occurs distal to this locus.

normal males (data not shown). While it is known that X inactivation may spread discontinuously and "skip" some genes and silence others located more distally,[19] evidence for the silencing of other genes surrounding *SRY* by a spreading of X inactivation was almost completely lacking. Expression analysis of translocated Y chromosome genes showed that the vast majority were normally expressed from the der(X;Y), apparently suggesting that the ability of X inactivation to spread into Y chromatin is relatively limited when compared to X;autosome translocations.[17–19] However, it should be noted that all of the genes we tested have X linked homologues which escape the spread of X inactivation, potentially biasing our results. Because of their high degree of sequence similarity, it might be predicted that these X/Y homologous genes, and in particular the PAR genes which are identical on the X and Y, would be resistant to the X inactivation signal. Thus our analysis may underestimate the true ability of X inactivation to spread through Y chromatin.

The inconsistency of our observations with the initial hypothesis led us to consider alternative explanations that could account for the occurrence of incomplete masculinisation in some cases of X/Y translocation. The clear association between translocation breakpoints in close proximity to *SRY* and the hermaphroditism observed in cases 1–3 suggested that disruption of sex determination might instead result from position effects interfering with normal *SRY* expression. In support of this hypothesis, a review of the literature revealed that almost all reported *SRY* positive 46,XX true hermaphrodites that have been studied molecularly have been found to have breakpoints very close to *SRY*.[6 8 33–35] Furthermore, previous studies in both man and mouse have found that deletions of sequences flanking *SRY*, some of which overlap with the location of the translocation breakpoints in cases 1 and 2, are associated with sex reversal,[36–38] providing strong evidence that position effects can disrupt *SRY* function.

Although the three other studied patients with incomplete masculinisation (cases 5, 7, and 14) all had translocation breakpoints distant from *SRY*, thus making it highly unlikely that the translocation breakpoint itself directly disrupts *SRY*, cases 7 and 14 both showed disrupted expression of *ZFY* (located ~170 kb proximal to *SRY*) in a manner unrelated to X inactivation. In case 7 *ZFY* expression was completely abolished despite the fact that the der(X;Y) remained active in a proportion of cells. Such constitutional inactivation excludes X inactivation as the underlying cause, and instead suggests inactivation resulting directly from genomic rearrangement. Similarly case 14 *ZFY* exhibited reduced expression despite the der(X;Y) being almost exclusively inactive.

Because of this combination of reduced *ZFY* expression attributable to position effects and intersex phenotype indicative of abnormal *SRY* expression, we hypothesised the presence of additional cryptic rearrangements within the translocated Yp11.31 region in these two individuals which disrupt both *ZFY* and *SRY* expression. Many apparently balanced translocations actually involve more complex rearrangements close to the site of breakage,[39–41] and a previous report described a Y;22 translocation associated with an interstitial deletion of *SRY* and sex reversal.[42] Consistent with this hypothesis, Southern blot analysis of distal Yp identified an abnormal hybridisation pattern in case 7, suggesting the presence of a cryptic rearrangement 3–8 kb proximal to *SRY* in this individual. As our PCR studies found no evidence for any deletion within this region, and *RPS4Y* showed apparently normal expression, this may represent an inversion within Yp11.31 with breakpoints close to *ZFY* and *SRY*. Although identical Southern analysis did not identify any abnormalities in cases 5 or 14, our analysis covered <25% of the ~170 kb region between *SRY* and *ZFY*, and thus the presence of cryptic rearrangements of Yp11.31 in these individuals cannot be excluded. Unfortunately, insufficient DNA from cases 7 and 14 and the very high density of repetitive DNA elements in this region precluded further such experiments. However, overall our data favour the suggestion that incomplete masculinisation in cases of X/Y translocation results from disruption of normal *SRY* expression by position effect, rather than X inactivation.

We also observed that a polymorphic Yp inversion[13 14] occurs significantly more frequently on Xp:Yp translocation chromosomes than in controls, confirming the observations of Jobling *et al* that the inversion predisposes to Xp/Yp translocation.[12] Furthermore, both the X and Y breakpoints on each der(X;Y) carrying the inversion were close to *PRKX* and *PRKY*, suggesting the inversion mediates illegitimate X/Y recombination by alignment of these homologous sequences during male meiosis. Consistent with this hypothesis, because *PRKY* is located within the inverted region it is brought into the same centromere to telomere orientation as *PRKX* in males carrying the Yp inversion (http://genome.cse.ucsc.edu/), thus aligning the X and Y homologues and providing an obvious mechanism by which the inversion mediates X/Y meiotic exchange. These results indicate that the Yp inversion polymorphism is an underlying factor in generating the *PRKX*/*PRKY* recombination hotspot observed in many XX males.[10 11]

These data represent the most detailed study to date of X/Y translocations, giving insight into the causes and effects of these rearrangements. Although we originally set out to study the spreading of X inactivation into Y chromatin, our results suggest that position effects disrupting gene expression also occur in a significant number of cases. When it is considered that we have only examined a relatively small number of genes on one side of each translocation breakpoint, we suggest that disrupted expression of genes in this way may be a relatively frequent occurrence, even in apparently balanced chromosomal rearrangements. In particular, we propose that incomplete masculinisation in cases of X/Y translocation is a result of disruption of normal *SRY* expression by position effect, rather than X inactivation.

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ELECTRONIC-DATABASE INFORMATION

The following URLs have been mentioned in this study: Human Genome Browser Gateway, http://genome.cse.ucsc.edu/; BLAST, http://www.ncbi.nlm.nih.gov/blast/; GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank; Webcutter 2.0, http://www.firstmarket.com/firstmarket/cutter/cut2.html; Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; and Locuslink, http://www.ncbi.nlm.nih.gov/LocusLink/.

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