Moderately repeated DNA sequences specific for the short arm of the human Y chromosome are present in XX males and reduced in copy number in an XY female

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Received 8 October 1985; Revised and Accepted 22 December 1985

ABSTRACT

Four DNA sequences specific for the Y chromosome were isolated from a recombinant phage library constructed from flow sorted human Y chromosomes. Two of these sequences were moderately repeated and assigned to the short arm of the Y chromosome by *in situ* hybridization. Both sequences were detected in five out of seven 46,XX males and were reduced in copy number in one out of two 46,XY gonadal dysgenesis patients tested. The findings suggest close proximity of these Y-specific moderately repeated DNA sequences to a testis determining locus.

INTRODUCTION

The human Y chromosome may be divided into two parts according to both cytogenetic and functional criteria. One part, the area between the tip of the short and the proximal long arm (Ypter->Yq1), excluding the centromere, contains early replicating euchromatic material as revealed by chromosome banding (1). Functional genes assigned to the Y seem to lie within this region (2). These genes include determinants required for normal testicular development, putatively, though not definitely, associated with the serologically detectable H-Y antigen (3,4,5); a gene for 12E7, an antigen defined by a monoclonal antibody (6); and genes for two Y-dependent polypeptides of unknown function (7). In addition, karyotype-phenotype correlations have prompted suggestions about Y-dependence of genes for stature (8), tooth size (9), spermatogenesis (10) and prevention of stigmata of Ullrich-Turner syndrome in males (2). The second part of the Y consists of the centromeric region and the constitutive, quinacrine bright heterochromatin, the latter comprising most of the Y long arm (11). Deletions of this last segment do not interfere with testicular differentiation, may, however, result in

infertility due to meiotic arrest (see Discussion in 12).

Molecular analysis of the Y reveals highly repetitive DNA (satellite DNA) in the nonfunctional segment of the distal long arm (13,14) and in the heterochromatin immediately adjacent to the centromere (15). Much of the long arm satellite DNA is composed of tandemly repeated Hae III restriction fragments of 3.4 kb and 2.1 kb and a relative copy number of 4000 and 2000 respectively (16). These repeated sequences are not cleaved by most available restriction endonucleases (17,18). Additional repeated sequences from the alphoid satellite DNA family are found in the pericentromeric region (15). Single copy or moderately repeated DNA sequences have been found in both the genetically active and inactive part. Many of these sequences are not purely Y-specific, but are also detected on the X chromosome or on autosomes or on combinations of both (19-26). The number of sequences found to be restricted to the Y is still low, and no function has of yet been unambiguously ascribed to any of them.

In order to learn more about the molecular structure of the Y and eventually detect functional genes, we constructed a recombinant DNA library from flow sorted human Y chromosomes. The library was enriched for the euchromatic part of the Y by cleaving the DNA with Hind III and cloning it into vector lambda Charon 21A, which has a maximum insert acceptable size less than 9 kb. The Hae III satellite DNA sequences, which for the most part lack Hind III sites (17,18), are not clonable in this phage. We then isolated Y-specific probes and further analyzed them by taking advantage of two genetic abnormalities interfering with normal gonadal development in humans, the XX male syndrome (27) and Swyer's syndrome (XY gonadal dysgenesis) (28). Individuals affected by the former syndrome are grossly normal but infertile males with a female karyotype, 46,XX. Conversely, Swyer's syndrome patients are phenotypic females with streak gonads and a male karyotype, 46,XY.

The XX male syndrome may be caused by abnormal presence of testis determining gene(s) in cytogenetic females. In fact, there are three types of evidence for the presence of Y chromosomal DNA in XX males. First, high resolution banding techniques demonstrate additional chromosomal material on the X of some XX

males (29,30). Second, studies in which Y-encoded antigen 12E7 was examined suggest X/Y interchange in paternal meiosis as one cause of the XX male syndrome (31). Third, Y-chromosomal DNA sequences are now being found in the DNA of several XX males (32,33). In Swyer's syndrome, Y chromosomal genes required for a normal testis to develop may be mutated or lost completely, as indicated by the finding of occasional Y chromosomal deletions (34,35,36). Thus, testing for the presence or absence of Y-chromosomal DNA sequences in these two disorders of gonadal development, i.e., 46,XX males and 46,XY females, should reveal sequences in close proximity to or identical with testis determining genes. Here we describe two moderately repeated sequences specific for the short arm of the Y that are both interesting from this perspective, since both were found in several XX males and both exhibited a drastically reduced copy number in one out of two 46,XY gonadal dysgenesis patients examined.

MATERIALS AND METHODS

1. Patients and fibroblast cultures: Fibroblasts from 7 XX males and from 2 XY gonadal dysgenesis patients were kindly provided by the following persons: Dr. W. Tilgen, Heidelberg; Dr. J.M.J. Scheres, Nijmegen; Dr. R.D. Wegner, Berlin; Prof. M. Fraccaro, Pavia; Prof. J. Lindsten, Stockholm; and Dr. J. Flori, Dijon. Routine cytogenetic analysis employing Q banding revealed a normal female, 46,XX karyotype in the 46,XX males and a 46,XY karyotype in the 46,XY females. Cytological evidence for chromosomal abnormalities in these cell lines was not found. The 49,XXXXY lymphoblastoid line (GM 1202) was obtained from the Human Genetic Cell Repository, Institute for Medical Research, Camden, NJ, and grown in minimum essential medium B supplemented with 10% fetal calf serum.

2. DNA panels and hybridization conditions: DNA was extracted from fibroblasts of patients and from white blood cells of 8 normal male and 8 female individuals as described (37). DNA was cleaved with the restriction endonuclease Hind III under conditions recommended by the manufacturer. DNA samples (2 ug/lane) were separated on a 0.7% agarose gel, transferred to

nitrocellulose filters (38) and baked in a vacuum oven for 2 hours. Filters were prehybridized in 4 x SSC, 0.05 M NaPO₄, pH 7.0, 8% dextran sulfate, 0.1% SDS, 0.001 M EDTA, 100 ug/ml tRNA, 25 ug/ml sheared and denatured salmon sperm DNA, 1 x Denhardt's solution, 50% formamide at 44°C overnight. Hybridizations with ³²P radiolabelled probes were carried out in the same buffer overnight at 44°C. Filters were washed in 0.1 x SSC and 0.1% SDS 7 times at room temperature and 3 times (20 minutes each) at 55°C. Patients as well as all 8 normal males and 8 normal females were investigated with Y specific probes Y-156 and Y-182. In addition, probes Y-202 and Y-97 were used for hybridization with DNA from all patients and from several male and female controls.

3. Construction of Y-enriched recombinant phage library:

A detailed description of the construction of the library is given elsewhere (Müller et al., in preparation). In brief: Chromosomes were isolated from a 49,XXXXY lymphoblastoid cell line according to Blumenthal et al. (39) and Sillar and Young (40). After being stained with a combination of Hoechst 33258 dye and chromomycin A3, chromosomes were analyzed on a dual laser FACS IV flow system (41). The chromosome fraction containing the Y was isolated, and DNA was extracted from it (41). The amount of DNA obtained, 50 ng of DNA from 5 x 10⁶ Y-enriched chromosomes, was estimated by comparison with known amounts of a DNA standard (42). Of this Y-enriched DNA, 40 ng was cleaved with 15 units of Hind III restriction endonuclease, ligated to 50 ng Hind III cleaved and calf intestinal phosphatase-treated Charon 21A phage DNA and packaged as phage (41,42). Most of the resultant phage, 5 x 10⁴ plaque forming units, was amplified on lawns of LE 392 bacteria, isolated, and stored in CsCl as described (41). However, 5 phage plates, containing approximately 5 x 10³ pfu, were immediately used for isolation of Y chromosomal DNA containing recombinant phage.

4. Screening of recombinant phage library:

Phage plaques were screened for human repetitive sequences according to Benton and Davis (43) as described (41). Phage that did not hybridize with total human DNA, radiolabelled by nick translation (44), were plaque purified and subjected to a second Benton-Davis analysis.

Phage plaques not hybridizing to human repeated sequences were used for the isolation of DNA as described (45). Isolated DNA was cleaved with restriction endonuclease Hind III in 50 μ l of "Hind III buffer" (prepared according to manufacturer's recommendations) and aliquots were separated on a 0.7% agarose gel. All hybridizations were done under conditions described above. Hind III cleaved DNA samples from phage containing human inserts were separated in a 0.6% low melting point agarose (IBI) gel containing ethidium bromide. Inserts were cut out on a UV lamp, dissolved in distilled water, and boiled for 7 minutes. After cooling to 37°C, aliquots were ^{32}P labelled applying an oligonucleotide labelling method of Feinberg and Vogelstein (46). The radiolabelled inserts were boiled and hybridized to nitrocellulose blots of genomic DNA samples to test for Y chromosome specificity. Probes found to be Y-specific were ^{32}P labelled and hybridized with panel filters containing DNA from 7 XX males, 2 XY gonadal dysgenesis patients and normal males and females.

5. Estimation of copy number: The approximate copy number of repeated Hind III restriction fragments was determined by diluting various amounts of a Y-specific phage insert plus pBR322, into which it had been subcloned (47), (corresponding to various copy numbers in the genome) with Hind III cleaved human female DNA (2 μ g per dilution). Insert and DNA were separated on a 0.7% agarose gel together with Hind III cleaved DNA from normal male controls. DNA was blotted on nitrocellulose filters and hybridized with the ^{32}P labelled probe under investigation. Densitometric comparison (48) of the hybridization signal obtained with the inserts with that of normal male DNA gave an estimate of the copy number of the restriction fragment in male DNA.

6. In situ hybridization: In situ hybridization was done according to Harper et al. (49), as modified by Donlon et al. (50).

RESULTS

Figure 1 depicts the flow histogram of the Hoechst 33258 plus chromomycin A3 stained chromosomes isolated from the 49,XXXXY lymphoblastoid cell line. 5×10^6 Y-enriched chromosomes were flow sorted and used as source of DNA for the construction of the

Figure 1. Bivariate flow cytometric analysis of 49,XXXXY chromosomes. Metaphase chromosomes were isolated, stained with Hoechst 33258 and chromomycin A₃, and analyzed using FACS IV instrument configured as described (40). The ordinate is a linear scale of Hoechst 33258 fluorescence intensity. The abscissa is a linear scale of chromomycin A₃ fluorescence intensity. Relative numbers of chromosomes are reflected in contour lines. Fluorescence peak assigned to the Y is indicated.

recombinant phage DNA library. Forty nanograms of DNA, representing 80% of the sample, was cleaved with Hind III, cloned in Charon 21A and processed as described in the Methods section. 50,000 phage plaque forming units were obtained from the packaging reaction. Based on an average insert size of 3.0 kb, this amounts to approximately three average representations of Hind III fragments from the human Y.

Recombinant phage that did not hybridize with repeated human DNA were isolated and tested for Y specificity of their inserts. Out of a total of 28 inserts tested and amenable (e.g. because they were either single copy or only moderately repeated) to mapping, fifteen inserts were found to hybridize with Y-chromosomal DNA sequences (Table 1). Eleven of the fifteen hybridized in addition to X and/or autosomal sequences. Four probes were Y-specific, hybridizing only with Y-containing

Table 1. Y-homologous DNA Probes from Charon 21A Library

Probe	Characteristics	Probe	Characteristics
Y-202	Y-specific unique	Y-7a	Multiple Y and non-Y homologies
Y-97	Y-specific repeated	Y-7b	
Y-156	" "	Y-7c	
Y-182	" "	Y-73a	
Y-157a	Y-single copy +	Y-73b	
Y-198	single copy, non-Y	Y-98	
	homology	Y-109	
Y-1	Y-single copy +		
Y-61	multiple, non-Y		
	homologies		

DNA. These probes were investigated further. Two of these probes (Y-182 and Y-202), hybridized with one single band of Hind III-cleaved, Y containing DNA. The molecular weights of the

Figure 2. Autoradiograph of hybridization pattern of probe Y-182 with Hind III digested DNA from normal, 46,XX females (lanes 1,12), normal, 46,XY males (lanes 2,3,4), 46,XX males (lanes 5,6,7,8,9,10,11) and 46,XY females (lanes 13,14). Identical amounts of DNA were applied in each lane. Note the strikingly reduced hybridization signal obtained with DNA from the 46,XX males and with one 46,XY female (lane 13) as compared to 46,XY male controls and gonadal dysgenesis patient 14.

Figure 3. Autoradiograph of hybridization pattern of probe Y-202 with Hind III digested DNA from two normal males (lanes 1,2) and two normal females (lanes 3,4,).

fragments were identical to the probe sizes (1.85 kb and 2.3 kb, respectively; Figs. 2,3). DNA complementary to probe Y-182 was repeated and had an estimated copy number of approximately 5 (see below) (Fig. 2). DNA complementary to Y-202 was repeated at most a few times per genome (Fig. 3). The other two Y-specific probes (Y-156 and Y-97) were also repeated and hybridized with several fragments of Y containing Hind III cleaved DNA (Figs. 4,5). The genomic DNA Hind III restriction fragments of the same sizes as the probes hybridized most strongly. Y-156 detected a pronounced band of 4.4 kb (corresponding to the size of the probe) together with 6 fainter bands of sizes ranging from 2 to 15 kb (Fig. 4). The most pronounced Hind III fragment hybridizing with probe Y-97 was 6.7 kb (Fig. 5). Molecular weight of the less intensely hybridizing fragment was 6.0 kb (Fig. 5).

All four Y-specific probes isolated were tested on panels containing DNA from seven XX males, two XY gonadal dysgenesis patients and normal male and female controls. Hybridization of two probes, Y-202 and Y-97 (Figs. 3,5), resulted in the same

Figure 4. Autoradiograph of hybridization pattern of probe Y-156 with Hind III digested DNA from normal 46,XX females (lanes 1,12), normal 46,XY males (lanes 2,3,4), 46,XX males (lanes 5,6,7,8,9,10,11) and 46,XY females (lanes 13,14). Identical amounts of DNA were applied in each lane. Note the strikingly reduced hybridization signal of the 4.4 kb fragment in gonadal dysgenesis patient 13 and the lack of several minor sequences of the Y-specific repeat in the XX males.

hybridization signals with DNA from normal males and from the gonadal dysgenesis patients, and no hybridization signal was found with DNA from normal females and XX males. Probes Y-156 and Y-182, however, hybridized with DNA from 5 out of 7 XX males in addition to normal male controls, testing the same amounts of DNA as of controls, and both resulted in weaker hybridization signals in one of the two XY gonadal dysgenesis patients (Figs. 2,4). Hybridization was not found with DNA from any of eight normal, 46,XX females. In addition, the hybridization signal of probe Y-182, that detects one single band, was weaker in the 46,XX males than in eight normal 46,XY control males (Fig. 2).

Both probes Y-156 and Y-182 evidently detect Hind III fragments that are several times repeated on the Y chromosome. Estimation of their copy numbers (see Materials and Methods) gave an approximate number, for the Y-156-complementary, 4.4 kb Hind III restriction fragment of 10, and an approximate copy number for the Hind III restriction fragment of Y-182 of 5. Furthermore, densitometric analysis of the intensity of the hybridization

Figure 5. Autoradiograph of hybridization pattern of probe Y-97 with Hind III digested DNA from two normal males (lanes 1,2) and two normal females (lanes 3,4).

signals also allowed the estimation of the copy number of the restriction fragments detected by the two probes in the patients. The reduced hybridization signal obtained with probe Y-182 in DNA from XX males reflects a copy number of 1 or 2 as compared to approximately 5 copies found in normal males. The reduced hybridization of probe Y-156 with the 4.4 kb fragment in one of the two XY gonadal dysgenesis patients suggests a copy number of 1 to 2 as compared to the approximately ten copies detected in normal male controls. Similarly, the copy number of Y-182 restriction fragments is reduced in the same patient and comprises approximately 3 copies.

In order to localize these probes on the Y chromosome, in situ hybridization was performed with probes Y-156 and Y-182. Metaphases prepared from lymphocytes of two normal males were evaluated, and grains above background level were only found on the short arm of the Y from both individuals. The highest concentration of grains was detected on the middle of Yp, corresponding to band Yp 11.2, with fewer grains towards telomere

Figure 6. In situ hybridization of Y-156 to a 46,XY derived metaphase. The probe was labeled to 2.3×10^7 cpm/ μ g, hybridized at a final concentration of 0.01 ng/ μ l at 42°C, and exposed for six days. Fifty-five percent of all metaphases examined showed at least one grain over Yp, as shown by the arrow. The insert shows the grain distribution obtained from 50 cells analyzed.

and centromere, respectively, as shown for one male (Fig. 6). The same probe was used on a second male, with identical results (32 cells, 27 grains detected over the Y, 19 of these over Yp). While the degree of intensity of hybridization was higher than that of most single copy probes, the degree of grain scatter appeared to be no greater than that found for single copy probes. Similar results were obtained with probe Y-182 (results not shown).

DISCUSSION

A recombinant phage DNA library, constructed from flow sorted human Y chromosomes was screened for Y-specific sequences. The library was enriched for non-satellite DNA from the Y. Under the assumption that the satellite DNA, especially the 3.4 kb and 2.1 kb tandemly repeated Hae III restriction fragments, make up 60% to 70% of the human Y (16), the Y chromosomal sequences in the present library primarily originate from those 30% that include

the euchromatic part and thus contain functional genes.

Four inserts isolated from this library detected sequences that are only on the Y chromosome. Three of these Y-specific sequences were moderately repeated and one was presumably single copy. Two probes (Y-156 and Y-182) detected Y-specific sequences in 5 out of 7 46,XX males. This, together with earlier findings of Y chromosomal sequences in some 46,XX males (32,33,51-54), corroborates the view that translocations of Y chromosomal DNA containing testis determining genes are a contributing cause of this syndrome.

All XX males studied in this investigation had a cytogenetically normal 46,XX karyotype, and evidence of extra chromosomal material was not found. Assuming that the smallest unit (metaphase chromosome "band") detectable in metaphase chromosomes by conventional staining techniques contains approximately 5 to 10×10^6 base pairs (450 band karyotype), the submicroscopic translocation from the Y that had occurred in these patients must comprise less than 5 to 10×10^6 base pairs.

The failure of the present studies and those of several others (32,33,51-54) to detect Y chromosomal sequences in all patients with XX male syndrome may have one of three explanations. The first is the possibility that there is considerable variation in the size of Y chromosomal translocations and/or insertions in 46,XX males. The second possibility is that Y-probe negative XX males are chimeras, containing a 46,XX and a 46,X,t(X;Y) cell line and that the cells used for the preparation of DNA contained the 46,XX cell line only. In accordance with this is the finding of 46,XX/46,XY and 46,XX/46,X der(Y) chimeric karyotypes in some cases of true hermaphroditism, a syndrome closely related to the 46,XX male syndrome. Third, it is conceivable that some cases of the 46,XX male syndrome are due to mutations of genes not on the Y chromosome. Support for this idea comes from the observation of familial cases of 46,XX male syndrome suggesting an autosomal recessive mode of inheritance (55). However, this inheritance may really be "pseudoautosomal" (56), due to Y sequences distal to an obligate X-Y short arm crossover. Consistent with this is the recent discovery of Y chromosomal material in familial 46,XX male cases (33), which makes an autosomal recessive mode of inheritance

and thus a non-Y related etiology of the XX male syndrome less likely.

While the major genomic DNA fragments detected by probe Y-156 (especially the 10-fold repeated 4.4 kb fragment) in normal males were present in the five 46,XX males, several minor bands (Fig. 4) were missing and evidently not involved in the Y chromosomal translocation. Assuming that sequence conservation is less towards the edge of a repeated domain (57), one may argue that the translocation event took place between conserved and less conserved sequences within this domain. In fact, our in situ finding of limited grain distribution supports the view that the repeats are not distributed over a large cytological region but do occur in a limited domain.

The 1.85 kb restriction fragment detected by probe Y-182 gave a weaker hybridization signal in 46,XX males than in normal males, thus indicating that not all copies of this repeat were translocated from a Y to an X or autosome in these males and that the translocation occurred directly within rather than towards the edge of the conserved repeats.

Our findings in one out of two XY gonadal dysgenesis patients further suggest close proximity of the two moderately repeated sequences to testis determining genes. In one of these gonadal dysgenesis patients, the copy number of sequences detected by probe Y-156 was reduced from 10 in normal males to 1 or 2. Furthermore, only half of the copies detected by probe Y-182 in normal males was present in the same patient. One could argue that the reduced copy number of these sequences reflects a quantitative polymorphism of these probes. This possibility, however, seems to be rather remote. No quantitative differences in these two repeats were found in 8 normal male controls. Also, both evidently unrelated probes detecting restriction fragments of different sizes, showed reduced copy numbers in the same patient. It is thus most likely that there is a microdeletion of the Y chromosome in this patient that involves part of the repeats, and, since gonadal differentiation is disturbed in these patients, testis determining genes. The DNA hybridization pattern in the second XY gonadal dysgenesis patient with probes Y-156 and Y-182 was not different from that detected in normal males. Therefore,

XY gonadal dysgenesis in this patient must be caused by either a point mutation affecting testis determining gene(s) or by a very small deletion not detected by our probes. As a result, different mutations (at the level of DNA segments) may cause gonadal dysgenesis just as different mutations (point mutation, deletion, etc.) may cause the same monogenic syndrome (e.g. 58,59).

In conclusion, we have described two moderately repeated sequences on the short arm of the Y that seem to be in close proximity of testis determining genes in 46,XX males and 46,XY females. These sequences seem to be the first purely Y chromosome-specific short arm repeats. Because of their Y-specificity, gains or reduction in their genomic representation can be detected not only with Southern blots, but with simpler DNA "dot blots" (data not shown). One "middle repetitive sequence", found on Yp before, is also present on the X and on autosomes (51) and required Southern blotting to study. The likely proximity of the purely Y-specific DNA sequences to a testis determining locus makes them especially valuable for the detection of functional Y chromosomal material in patients with disorders of primary sex differentiation. As such, they may be an ideal, easily utilizable tool for the investigation of genes involved in testicular differentiation.

ACKNOWLEDGEMENTS

We are grateful to Professor Ulrich Wolf, Freiburg, who collected the fibroblast cultures used in this investigation, for sharing the cells with us. We also acknowledge Alan Flint for his skillful help with chromosome sorting and Anthony Monaco and Louis Kunkel for critically reading the manuscript. Ulrich Müller is a recipient of a Heisenberg fellowship from the Deutsche Forschungsgemeinschaft. This work was supported by research grants GM33579 and HD18658 from the National Institutes of Health and 1-353 from the National Foundation March of Dimes.

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