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5.0 DISCUSSION

This study endeavours to define the molecular organization and evolution of sequences related to the long arm of human Y chromosome. A sizable amount of data is available on almost all aspects involving other chromosomes, whereas, Y chromosome has been rather difficult to deal with, mostly because of its exclusive nature observed at meiosis. In the present study, attempts have been made first to understand most commonly occurring repeat sequences on the long arm of human Y chromosome and then use them as probe to detect the Yq material in the normal humans as well as in the patients suffering from sex chromosome anomalies. The experimental approach with respect to design and development of oligonucleotide probe is based on absolute complementarity exhibited by oligonucleotide probes. Unlike the cloned probes, under appropriate conditions synthetic oligonucleotide probes display essentially absolute hybridization specificity. However, the hybridization specificity vary when there are one or more non-Watson-Crick base pairs. Of the eight non-Watson-Crick base pairs (CC, AA, TT, GG, CA, CT, GA, GT), the dissociation profiles of six of them have been studied in Southern hybridization. Four of them (CA, AA, TT, CT) were found to dissociate five times faster while two of them (GA, GT) 2.5 times as compared to perfectly matched duplexes (Itakura et al., 1984). During probe design this dissociation profile is of importance and forms the basis of stringency conditions for a particular probe (Miyada and Wallace, 1987).

In the present study, besides synthetic oligonucleotide probes, genome derived fragment has also been used to compare the respective hybridization patterns.
5.1 Southern Hybridization With Genome Derived Fragment-probe

The DYZ1 fraction, one of the major repeating units present on the long arm of the human Y chromosome, was reported by Cooke (1976). Other workers (Bostock et al., 1978; Nakahori et al., 1986) have reported the presence of cross-hybridizing sequences in the DYZ1 fraction that form part of the satellite II and III repeat sequences. When used as a probe, DYZ1 would be expected to detect a male-specific hybridization pattern. The results of the present study are in accordance with this postulation since a male-specific hybridization pattern was obtained when DYZ1 fraction was hybridized against Hae III digested human male genomic DNA. Further, the presence of additional bands in the high molecular weight region both in male and female indicates that they must be contributed by autosomes. This confirms the earlier findings of Bostock et al. (1978) and Nakahori et al. (1986). However, a smear in the lower molecular weight region was also obtained which could probably be due to the fact that the fragment-probe being a gel-eluted fragment contains other sequences that migrate at the size 3.4 kb and cross-hybridize partially with the sequences in the lower molecular weight region.

These results suggested that genome derived uncloned fragment used as probe for detecting Yq material may not be the right choice because of additional non-specific signals. This led us to explore the possibility of developing pure synthetic DNA probes deduced from the sequences of the DYZ1 contig.

5.2 Optimization of sequence length and complexity of the oligonucleotide probe in Southern hybridization

The factors playing a major role in oligonucleotide hybridization include length of the oligonucleotide (Wallace et al., 1979), (G + C) content (Schulze et al., 1983) and the
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Temperatures for hybridization and post-hybridization wash conditions (Wallace et al., 1979; Miyada et al., 1985). Stringency conditions for the set of oligonucleotides used in this study are given in the section, Materials and Methods. Another lesser known factor affecting oligonucleotide hybridization with the immobilized target DNA, is the kinetics of the hybridization which is essentially first order with respect to the oligonucleotide concentration (Wallace and Miyada, 1987). These factors were taken into consideration while using oligonucleotide probes in Southern hybridization.

5.2.1 Oligonucleotide probes based on 5'TTCCA 3' motif

The length of the oligonucleotide probe determines its hybridization specificity. The longer a sequence, the more likely it is to be unique among the collection of sequences and lesser would be the chances that it will bind non-specifically to other sequences (Miyada et al., 1985). As expected, of the five probes, OAT10Y, OAT15Y, OAT20Y, OAT25Y and OAT30Y used, OAT10Y, comprising 2 units of 5'TTCCA 3', was found to be unstable at the lowest stringency conditions possible, indicating that the probe does not fulfill the optimal length criterion and dissociates at high stringency conditions. Obviously OAT10Y was not considered as the potential probe for the detection of Yq material. The other four oligonucleotides, namely, OAT15Y, OAT20Y, OAT25Y and OAT30Y were found to be equally informative but OAT20Y turned out to be most credible probe, detecting a stable, high intensity, male-specific 3.4 kb band.

Computer search using DNAsis software (Table 2) showed that the pure repeats of 5'TTCCA 3' are not repeated more than three times in the DYZ1 fraction but the autoradiographic signals are best obtained with OAT20Y containing four consecutive repeats of 5'TTCCA 3'. The contradiction observed can be explained in terms of the target sequences with three pure repeats of 5'TTCCA 3' and additional sequences at the
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3' and/or 5' ends. Alternatively it may also be possible that other 3.4 kb fraction may have more than three consecutive repeats of 5'TTCCA 3'. This phenomenon in turn would lead to the increased intensity of the autoradiographic signals, as was observed with the oligonucleotide OAT20Y. More than four pure repeats of 5'TTCCA 3' would also increase the number of mismatches observed, resulting in destabilization of the target-probe hybrid as was observed with OAT25Y and OAT30Y.

The chemistry of oligonucleotide hybridization dictates the formation of only perfect Watson-Crick base pairs at high stringency and sequences with even a single mismatch are destabilized. The hybridization profiles of the OAT20Y probe and the DYZ1 fragment-probe revealed that OAT20Y was a better probe though with respect to sex-specific hybridization pattern, both the probes revealed similar results. This property of oligonucleotide hybridization has been successfully used earlier in the detection of base substitutions between two alleles, most significantly, in diagnosis of genetic diseases like sickle cell anemia (Conner et al., 1983; Studencki and Wallace, 1984; Murasuqi and Wallace, 1986).

5.2.2 5' TTCCA 3' motif versus its variations

To characterize different versions of 5'TTCCA 3' motif distributed in the genome, the hybridization patterns of oligonucleotides, OAT25YU, OAT20Y, GS20.1Y and GS20.2Y against genomic DNA digested with enzymes, Hae III and Eco RI were assessed. The rationale is based on the fact that of the several probes tried, some may be unique to Yq and hence the signal contributed exclusively form the Yq could be detected. Of all the four oligonucleotide probes used, signals were detected in lower and higher molecular weight region but none of the probes revealed Y chromosome unique pattern. Since additional bands were obtained in all the samples, in the higher
molecular weight region with Hae III and lower molecular weight region with Eco RI, they are obviously contributed by the autosomal fraction of non-Y-specific 3.4 kb repeats.

Since the probe OAT25YU carried a non-Watson-Crick base pair in every one of its single unit, it was expected to detect a fairly unstable hybridization pattern. True to this prediction, OAT25YU showed a total loss of the male-specific band at the 3.4 kb region against Hae III digested genomic DNA even though the motif, 5'TTCGA 3', carried within OAT25YU, is the second most frequently occurring motif in the DYZ1 fraction. On the other hand, the signals in the higher molecular weight region, though faint, were retained. This observation signified the predominance of 5'TTCCA 3' motif in the Y-specific DYZ1 fraction. Furthermore, presence of a CA base-pair in the middle of the oligonucleotide sequence would be expected to add to the destabilization of the hybrid duplex at high stringency. This fact was confirmed by the hybridization study of the probe, GS20.1Y. A substitution (C → A) at the 9th position, formed rather unstable target-probe duplexes at the male-specific and autosomal regions and detected low intensity signals at both the regions with the probe, GS20.1Y. The other oligonucleotide, GS20.2Y with two non-Watson-Crick base pairs (CT and GT) would be expected to destabilize the hybrid duplex at rates higher than that observed with GS20.1Y. The GS20.2Y did form unstable hybrid duplex at the male-specific region but detected high intensity signals at the high molecular weight region. This diametric result drew attention to the previously noted observation of the absence of the variations of 5'TTCCA 3' motif in the Y-specific DYZ1 while their presence in the non-Y-specific 3.4 kb repeat. Additionally, on the basis of the results obtained, it is postulated that such variations form a sizable portion of the autosomal fraction such that a high intensity signal in the high molecular weight region is obtained with the
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oligonucleotide probe, GS20.2Y, that harbours two base substitution. Variations of 5'TTCCA 3' motif seem to be absent from the Y-specific DYZ1 fraction and thus contribute to the preferential loss of male-specific signals, as observed with OAT25YU and GS20.2Y. Two additional oligonucleotides, GS17Y and GS18Y with three pure repeats of 5'TTCCA 3' and additional sequences at the 3' end, TT and TTC, respectively, were also tested in the hybridization for the stability of resultant hetero-duplex. Hybridization patterns of OAT15Y, GS17Y and GS18Y showed a male-specific band and autosomal bands with the enzymes, Hae III and Eco RI. The hybridization with OAT15Y and GS18Y uncovered comparable intensity signals at the 3.4 kb region thereby indicating the additional stability imparted by cytidine residue at the 3' end to the hetero-duplex in case of GS18Y such that a pattern better than OAT15Y is detectable. This also resulted in GS18Y detecting signals in the high molecular weight region. GS17Y, on the other hand, detected faint male-specific signal while signals in the autosomal region were not accordingly reduced. This preferential loss of male-specific signal indicates the variations of the motif 5'TTCCA 3' being either absent or forming a negligible proportion of the DYZ1 fraction while their preponderance in the autosomal fraction. With Eco RI digested DNA, in addition to the male-specific signals at the 3.4 kb region, the probes detected monomorphc signals in the lower molecular weight region. These monomorphic bands were detected better with GS17Y and GS18Y than with OAT15Y. Further, if the assumption that variations of TTCCA are present in the autosomal fraction of 3.4 kb repeat holds true then the detection of high intensity monomorphic bands with GS17Y and GS18Y against Eco RI digested DNA and preferential loss of male-specific signal with GS17Y against Hae III digested DNA can be explained. In conclusion, these results provide evidence that in contrast to non-Y-specific sequences, Y-specific fraction of 3.4 kb sequences
that is, DYZ1 fraction, has fewer variations of 5' TTCCA 3' present.

5.3 Polymorphic Information Content of the probe, OAT20Y

The sequences on the Y chromosome have been postulated to move rapidly in an intra- as well as an inter- chromosomal environment. Thus these sequences seem to have evolved rapidly as opposed to autosomal and X-chromosomal sequences which exhibit relative evolutionary stability due to linkage (Buckle et al., 1984). The degree of variation in 5' TTCCA 3' motif, in terms of copy number and sequence, in different individuals was tested by employing conventional RFLP approach; digesting DNA from unrelated human male samples with \textit{Hae} III and hybridizing with OAT20Y. Copy number variation could be seen in the intensity of the 3.4 kb band, while specificity of the oligonucleotide hybridization was made use of to comment upon sequence variation. A uniform 3.4 kb isomorphic band was obtained in all the samples tested. Of all the individuals tested, one (#3, Fig. 3b) showed lower intensity of the 3.4 kb male-specific band indicating a copy number variation of these repeats on the Y chromosome of this individual. This observation is in conformity with the earlier findings where the long arm of Y chromosome has been shown to be polymorphic in terms of the copy number of these repeats which varies from 800-4000 copies in normal individuals (Kunkel et al., 1977; Cooke & Mckay, 1978; Cooke and Hindley, 1979; Lau, 1985; Nakahori et al., 1986). The uniformity of signal intensity at the 3.4 kb region in most of the samples indicated very little sequence variation in the motif, 5' TTCCA 3'. However, when oligonucleotide probes based on the variations of 5' TTCCA 3' were used in Southern hybridization, a perceptible loss of signal only at the male-specific 3.4 kb band was observed. Thus, 5' TTCCA 3' would seem to form a major portion of DYZ1 fraction in the individuals tested and few individual-specific
changes in terms of the base sequence and length have occurred. These results also provide conclusive evidence that the Y sequences exhibit evolutionary stability in an intra- as well as inter- chromosomal environment.

Recent observations by Strand et al. (1993) suggest that in an in vivo scenario, DNA polymerase has a considerably higher rate of strand slippage on DNA templates containing simple repeats. Most of these errors are corrected by cellular repair machinery but many such errors are responsible for the loss or gain of a repeat motif observed as a result of defective post-replicational hetero-duplex repair. This observation implies that simple repeat motif when used as probe against an informative enzyme, will possibly detect polymorphism. The results of the present study do not support this hypothesis since OAT20Y did not detect polymorphism by conventional RFLP approach in the human genome although a wide spectrum of restriction enzymes were used for the digestion of genomic DNA. On the contrary, consistently monomorphic hybridization pattern was observed when human genomic DNA digested with sixteen different enzymes was probed with OAT20Y. This implies that a very effective post-replicational repair mechanism which corrects the faults of strand slippage, gives correct alignment to the DNA strands and thus maintains the copy number of these repeat sequences. Alternatively, these sequences, contrary to the common belief, donot serve as the hot spots for recombinations and are located in silent region of the genome. The fact that OAT20Y gives universal monomorphic pattern in all the individuals tested also implied that these repeats have become amplified en-bloc and the DNA fragment in which these repeats are buried remains unaltered in different individuals. But Yokoi et al. (1989) have reported detection of low level polymorphism \((10^{-6})\) of autosomal origin using nick-translated pHY10 (Nakahori et al., 1986) which carries one unit of DYZ1 against Taq I digested human genomic
DNA. 5meC has been shown to mutate at higher frequency than normal in bacteria and serves as the mutational hot spot (Miller, 1983). In analogy, Wallace et al. (1985) proposed 5meC residues in the dinucleotide CpG to be the mutational hot spots in mammalian cells as well, such that deamination of 5meC to T would result in the loss of Taq I and Msp I restriction site. Thus, chances of detecting polymorphism would be more with Taq I and Msp I than with the other enzymes. OAT20Y when used as probe against Taq I and Msp I digested human genomic DNA detected bands which were not clear due to a smeary background. Although a wide spectrum of restriction enzymes have been used to establish the PIC of the probe, it seems highly unlikely to detect polymorphism by hybridizing genomic digests with OAT20Y using other restriction enzymes.

5.4 Evolutionary conservation of the repeat motif, 5'TTCCA 3'
Kunkel and Smith (1982) have described Y-specific 3.4 kb Hae III (DYZ1) sequences that do not cross-hybridize with either human female or ape DNA and thus seem to be a rather recent inclusion in the human Y chromosome. In these Y-specific sequences, 5'TTCCA 3' is the most abundant repeat motif. The evolutionary conservation of 5'TTCCA 3' motif was studied by evaluating the presence of this motif in the DNA of non-human genomes. On hybridizing Hae III digested DNA from different species with OAT20Y, the results of high stringent hybridization revealed human-specific autoradiographic signals. On the other hand, low stringency hybridization uncovered negligible smeary signals in rhesus and langur, in addition to distinct human-specific signals. Thus, like DYZ1, 5'TTCCA 3' also exhibits species specificity. These results are not in accordance with the earlier findings of Grady et al. (1992) who reported a signal in human as well as non-human species (Orangutan, Chicken, Maize, Drosophila,
Sea Urchin and Yeast) using [CAACCGAGT(GGAAT)₆] as probe. However, it is not possible to make a verbatim comparison of the results reported in the present study with those of Grady et al. (1992), as the oligonucleotide probe used by them has flanking sequences in addition to the focal sequence which is complementary to the motif 5'TTCCA 3'. Moreover, the non-human species tested by them have not been included in this study. It may be argued that the absence of signal in non-human species in the present study could be due to the cleavage of internal sites complementary to 5'TTCCA 3'. This possibility was ruled out by zoo-dot-blott hybridization. Undigested DNA samples from human and non-human species, hybridized with OAT20Y, also yielded human-specific signals. This result further strengthened the belief that the 5'TTCCA 3' motif is species-specific with respect to humans.

Ganguly and co-workers (1992) have reported a Bam HI repeat element prominently associated with the neo-Y chromosome of Drosophila miranda while being absent from genome of Drosophila melanogaster. In humans too, insertion of a Y-specific Alu I repeat, YAP (Y Alu Polymorphism), has been reported in human evolution, sometime before the divergence of modern human group (Hammer et al., 1993). In analogy to these observations, absence of this motif from the monkey species tested, indicated that these sequences have evolved independently and rather recently, probably after the divergence of humans from non-human primates.

5.5 Sensitivity of the probe, OAT20Y

OAT20Y is based on a repeat motif 5'TTCCA 3' present in the DYZ1 fragment, localized on the long arm of human Y chromosome which constitutes about 40% of the total Yq material. Due to the overabundance of this repeat fraction on the Y, cloned probes based on DYZ1 have been used for the detection of the Y chromosome
material by earlier workers (Cooke et al., 1982; Lau, 1985; Sandberg, 1985; Kobayashi et al., 1988; Nakagome et al., 1991). The present work, to our information, reports for the first time, detection of Y chromosome material using an oligonucleotide probe based on the repeat motif, 5'TTCCA 3' (already published, Ali et al., 1992). Discernible male-specific band was obtained with as little as 1:20 (5%) of male DNA in female control DNA. Though other workers (Yokoi et al., 1989; Witt et al. 1993) have reported better efficiency (700 pg and 100 pg, respectively) using cloned probe in Southern hybridization and PCR technique using Y specific primers, the specificity and ease of oligonucleotide hybridization would certainly be an advantage over the existing methods of detection.

5.6 Detection of Yq chromosomal material with OAT20Y probe

Methods of molecular determination of sex, using Y-specific probes, are useful not only for prenatal or postnatal diagnosis of sex linked disorders but also for detection of residual host cells in patients receiving bone marrow transplant (BMT) from a donor of the opposite sex (Lawler et al., 1989; Amtonucci et al., 1993), for the detection of 45,X/46,XY mosaicism in Turner's syndrome patient and for determining structural aberrations of the Y chromosome (Stalvey et al., 1988; Witt et al., 1993). The detection of Y chromosome material in females with dysgenetic gonads and a Y chromosome has been of paramount importance due to high incidence of the development of gonadoblastoma in them (Teter et al., 1964; Robinson et al., 1964; Strumpf, 1965; Borghy et al., 1965; Teter and Boczkowski, 1967; Teter, 1969; Andrews, 1971; Schellhas, 1974; Manueal et al., 1976; Williamson et al., 1976; Fournier et al., 1976; Tykus et al., 1984). The integrity of Y chromosome, and in particular the presence of the distal fluorescent band (Yqh), has been the required
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criterion for the tumour development. This observation has been reinforced by the absence of gonadoblastoma in a Turner patient with a mosaic karyotype 45 X/46 XYnf (XY non-fluorescent), in whom the distal fluorescent band seen in normal Y chromosome was missing (Kofman et al., 1981; Ganshirt-Ahlert et al., 1987), and the presence of a normal, distally fluorescent Y in the karyotype of mosaic a Turner patient who developed the tumour in her dysgenetic gonad (Lukusa et al., 1986). Thus, an oligonucleotide probe detecting the presence of Yqh is postulated to contribute to an early and accurate detection and treatment of gonadoblastomas in such patients.

In the present study the presence of Yqh was ascertained using OAT20Y, the probe detecting male-specific hybridization pattern. The data obtained was further augmented by performing chromosomal banding on the cultured cells of the patients. A total of twelve patients were tested at the molecular level and at the cytogenetic level during the course of this study. Of these twelve patients, nine showed the clinical symptoms of Turner's syndrome, two were suffering from male hypogonadism while one patient showed the symptoms of male pseudohermaphroditism. Of the nine patients with proposed Turner's syndrome, tested in the present study, DNA from two individuals (P2 and P4, Fig. 14a) showed the presence of 3.4 kb male-specific band in Southern hybridization with OAT20Y. Chromosomal analysis showed the presence of an intact Y chromosome in both the cases. This analysis showed that OAT20Y is sensitive enough to detect Yqh material in individuals displaying mosaic karyotype. It is envisaged that OAT20Y would thus, prove to be of significance in the diagnosis and further treatment of such patients. Even in the absence of a cytogenetically detectable Y chromosome, presence of cryptic Y material has been predicted. The probe, OAT20Y, on the basis of present results, is believed to be sensitive enough to detect Y chromosome in such cases too. Development of gonadoblastomas in the apparent
absence of Y-chromosomal material has been reported in few cases where presence of Y chromatin has been excluded by conventional cytogenetic analysis, but not by DNA hybridization. This again emphasizes the importance of DNA analysis, as in many 46,XX males and 46,XY females, where Y-chromatin was not detected by cytogenetic analysis, could be detected by Y-specific DNA hybridization (Page, 1987).

Nagafuchi et al. (1992) mapped GBY to the centromeric portion of Y chromosome on the basis of detailed study of DNA from a Turner's syndrome patient (1514) who developed gonadoblastoma in her dysgenetic gonads. Though Page (1987) postulated GBY to be present on the interval 4B-7 which includes centromere and whole of the long arm, development of gonadoblastoma in the absence of Yqh has not yet been reported. Rather, researchers have emphasized on the integrity of Yqh for the development of gonadoblastoma.

Lau and Schonberg (1984) and Manz et al. (1992) have reported normal male individuals with apparent absence of Yq heterochromatin at the cytogenetic level. When tested at the molecular level with specific probes, the probands tested positive for the presence of DYZ1. On the basis of these results, these workers postulated putative role of DYZ1 sequences in normal male development. In the present series, the status of DYZ1 fraction was examined in three patients showing clinical symptoms of the disorders of male sexual development. They were tested for the presence of DYZ1 at the molecular and cytogenetic level. All three of them were positive for DYZ1 with OAT20Y hybridization. Chromosomal analysis on two of them (P1 and P12) showed clear presence of an intact Y chromosome. The results obtained indicated that though DYZ1 fraction might be normal in these patients, the clinical symptoms exhibited by them, possibly, are the manifestations of other physiological abnormalities.

Thus, on the basis of these results, it is envisaged that the oligonucleotide, OAT20Y
would prove to be a versatile probe in the detection of the long arm of the human Y chromosome in sex-chromosome related anomalies.

Similarly, in other cases of abnormalities of sex chromosomes, for example, in patients with small and structurally abnormal Y derived marker chromosomes, the identity of the marker may be difficult to determine by classical cytogenetic methods. However, the application of molecular genetic techniques combined with cytogenetics may greatly enhance the speed and accuracy of such diagnosis and may also be used retrospectively to confirm or negate previously made diagnosis (Witt et al., 1993).