Moderately repeated mouse Y chromosomal sequence families present distinct types of organization and evolutionary change

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ABSTRACT

Male specific repeated sequences compose approximately 10% of the mouse Y chromosome. This was deduced from studies of genomic phage clones that contain male specific DNA, and three subcloned EcoRI fragments pBC10-0.6, pBC15-1.1, and pBA33-1.8. Southern analyses and in situ hybridization of metaphase chromosomes show these three sequences to be present in 100-200 copies on the Y chromosome and in female DNA as single copies. One of these, pBC10-0.6, was directly demonstrated to be on the X chromosome. All three exhibited male specific transcription.

These sequences are found in two distinct organizations. Subclone pBC10-0.6 is uniformly found in a long HpaI repeat unit of 9.7 Kbp. Subclones pBC15-1.1 and pBA33-1.8 are distributed throughout several EcoRI repeat families in which the male specific fragments are interspersed. These male specific fragments also differ among themselves with regard to the types and frequency of evolutionary changes.

INTRODUCTION

The Y chromosome is the only one whose principle function appears directed toward a single biological process - that of male sex determination and gametogenesis (1,2). The Y chromosome is divided into two different parts. One part, toward the telomere, is homologous to the X chromosome and is responsible for meiotic pairing. The pairing region of the Y has been shown in humans and mice to contain DNA sequences and a protein locus whose genetic behavior are autosomal (3,4,5). They are present on both chromosomes as homologous copies and are exchanged during meiotic recombination. Consequently, the segregation of loci in this region does not correspond to that of traditional sex linked traits.

The sequence composition of the rest of the Y chromosome, toward the centromere, prevents homologous pairing and recombination with other chromosomes. Presumably this region contains no essential information and codes primarily for those products necessary for development of the male phenotype. The Y chromosome is the only one of which the major fraction is haploid in both

somatic cells and premeiotic germ cells.

Meiotic translocation of a small part of the Y chromosome to the X chromosome yields phenotypic males of the XX constitution in the progeny. Such individuals are designated XX^{SXR} for the sex reversed phenotype (6,7). This translocation enables a more defined identification of the testis determining part of the Y chromosome.

We and other laboratories have initiated studies of cloned DNA sequences from the mouse Y chromosome (7,8,9,10,11,12). We have isolated clones containing male specific DNA mixed with repeated sequences on other chromosomes (10). We expect male specific DNA to be present primarily in the nonpairing region, although parts of the pairing region may also contain male specific DNA. Studying male specific sequences from both of the recombinationally distinct regions of the Y chromosome provides the opportunity of comparing the roles of intrachromosomal and interchromosomal recombination in the generation of sequence diversity.

This paper describes several families of repeated sequences found predominantly on the Y chromosome. These rapidly evolving families are interspersed with each other and with repeated sequences also present on other chromosomes. These repeated sequences are distributed over only about 10 percent of the mouse Y chromosome.

MATERIALS AND METHODS

(a) Animals

Most inbred strains of mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The consomic 129/Sv (N13) strain carrying the Y chromosome from *Mus poschiavinus* were provided by Dr. J. Barry Whitney, III (Medical College of Georgia, Augusta).

(b) Clones

Bacteriophage clones containing male specific DNA were identified in a Charon 4A lambda library derived from BALB/c sperm by procedures already described in detail (10). Subclones were produced from EcoRI digested bacteriophage DNA ligated into the EcoRI site of pBR325 (13). The individual plasmid subclones are designated according to a) the particular phage from which the insert was derived, and b) its size. Thus, pBC10-0.6 is a plasmid (pB) subclone of a 0.6 Kbp fragment from bacteriophage C10.

(c) DNA Extraction and Analyses

Liver DNA from overnight starved mice was extracted as described (14). Plasmid DNA was labeled by nick translation (15) or by replacement synthesis of isolated inserts (16). Restriction endonuclease digestions were carried out

according to directions provided by the suppliers (usually Bethesda Research Laboratories). The resulting DNA fragments were separated on agarose gels and transferred to nitrocellulose filters according to Southern (17). Hybridizations were carried out at 42°C in 50% formamide (18).

(f) In situ Hybridization

Metaphase chromosome spreads from bone marrow cells were hybridized (19) with isolated plasmid inserts labeled with ^3H -dCTP (26 Ci/mmol) and ^3H -dATP (14 Ci/mmol). The chromosomes were stained with Giemsa (Harleco) diluted 1:10 with phosphate buffer pH 7.4. The time of autoradiography at -70°C was 6-10 weeks. Removal of the autoradiographic grains was accomplished by treatment of the slides for 1 min in 7.5% potassium ferricyanide followed by 2 min in 20% sodium thiosulfate (pentahydrate). Afterwards the chromosomes were restained with Giemsa.

(g) RNA Isolation and Analysis

Total RNA was isolated with the guanidinium thiocyanate method (20). PolyA⁺ RNA was electrophoresed (21) and probed on nitrocellulose filters as for Southern analysis. Dot blot analysis of total RNA was carried out as described (22).

RESULTS

Previously (10) we described a procedure for isolating genomic clones containing male specific DNA by screening plaques with labeled male DNA after all non male specific sequences have been excluded by extensive prehybridization with unlabeled female DNA. Application of this procedure to a charon 4A lambda library derived from BALB/c sperm DNA yielded a number of distinct clones containing male specific DNA. All of the clones isolated by this method contain both male-specific sequences and sequences shared with female DNA (10).

Several such genomic clones were chosen for further study. In Fig. 1 is a Southern blot of EcoRI digested genomic clones hybridized with ^{32}P -labeled male DNA. Fragments yielding intense signals contain highly repeated sequences common to both male and female DNA. Prehybridization of similar blots with female DNA markedly reduced or eliminated the reactivity of these bands (10). Other fragments on the same blots generated low intensity or no signals (Fig. 1) and these were candidates for sequences present predominantly in male DNA.

EcoRI digested genomic phage clones (numbered C10-I11) were subcloned into the EcoRI site of pBR325. The subclones were named according to the phage from which they were derived and the size of the insert in Kbp. Three of these, pBC10-0.6, pBC15-1.1, and pBA33-1.8, which fail to hybridize with each other (Fig. 1) were used to probe Southern blots of EcoRI-digested female and male

Figure 1. EcoRI fragments of five genomic phage clones (C10, C15, A33, H28, and I11) that contains male specific DNA (10). Each was digested with EcoRI, electrophoresed through agarose and transferred to Southern blots. In the left panel is the ethidium bromide stained gel. Indicated are the individual EcoRI fragments that were subcloned and studied further (see Methods for nomenclature). The three fragments pBC10-0.6, pBC15-1.1 and pBA33-1.8 show predominate male specificity, whereas pBH28-1.4 reacts with DNA from both sexes, albeit more intensely with male DNA. Sequences homologous to pBA33-1.8 are found in pBC15-9.4 and pBI11-2.9, also shown. The topmost two fragments of 18.0 and 11.9 Kbp are the arms of the lambda Charon 4A vector. In the other three panels are results of probing Southern transfers of the gel or a similar one with male DNA or DNA from the subclones pBA33-1.8 and pBC10-0.6.

mouse genomic DNA's (Fig. 2). All three probes gave intense signals with male DNA whereas little reactivity was observed with female DNA (Fig. 2). Under more sensitive hybridization conditions these probes were also observed to react with female DNA. This aspect is treated in more detail below.

DNA from sex reversed (XX^{SXR}) mice (6) was also tested for hybridization with our male specific clones. No hybridization beyond that observed in normal female mice was detectable (data not shown). Thus, these sequences are not localized to the testis determining part of the Y chromosome defined by the Sxr translocation.

The essential male specificity of clones pBC10-0.6, pBC15-1.1, and pBA33-1.8 would logically place them on the Y chromosome. This was directly demonstrated for pBC15-1.1 by in situ hybridization of metaphase chromosomes with 3H -labeled probes. Nests of grains were observed clustered over the Y chromosome identified by its small size and distinction from chromosome 19

Figure 2. Hybridization of subclones pBC10-0.6, pBC15-1.1, and pA33-1.8 with male (M) and female (F) DNA from an inbred strain (BALB/c) of laboratory mice, and from *Mus poschiavinus* (Posch). DNA's were digested with EcoRI, electrophoresed through 0.8% agarose, blotted and hybridized with nick translated probes.

(Fig. 3). *In situ* hybridization with pBA33-1.8 gave a similar accretion of grains over a single small chromosome. There does not appear to be a preferential localization of the grains on any particular part of the Y chromosome.

To determine the copy number in males of the major EcoRI fragment of pBC10-0.6 Southern blots were prepared from mixtures of known amounts of EcoRI digested pBC10-0.6 plasmid and female DNA's. Resulting hybridization intensities with the pBC10-0.6 probe were compared densitometrically with that given by a standard amount of EcoRI digested male DNA and yielded a copy number of about 90 per genome. The other two male specific fragments pBC15-1.1 and pBA33-1.8 are more dispersed and the sum of their hybridization signals relative to pBC10-0.6 yielded copy numbers of about 170 and 130, respectively, per genome.

I. Structure and Organization of the Male Specific Sequences.

We find that there are two distinct manners in which these male specific sequences are organized. One organization is that of pBC10-0.6. Southern

Figure 3. Autoradiographs of *in situ* hybridization of ^3H -labeled pBC15-1.1 (a) and pBA33-1.8 (b) with metaphase spreads. In these and other metaphases clusters of silver grains are localized to a single small chromosome which becomes visible after the grains have been removed [solid arrow, (b) and (d)]. In the upper metaphase this small chromosome is clearly distinguishable from the pair of chromosome 19's (open arrows) and therefore must be the Y chromosome.

blots of EcoRI-digested male genomic DNA probed with pBC10-0.6 gave intense hybridization with an equivalent size fragment (Fig. 2) termed the "cognate fragment" here. In these blots the hybridization signal is predominantly localized to the cognate fragment and with only minor signals detectable on other fragments. Our evidence indicates that pBC10-0.6 is part of a much longer repeated sequence. Thus, in blots of HpaI-digested male DNA the predominant hybridization signal is to a 9.7 Kbp fragment (data not shown). A

site for HpaI is not present in pBC10-0.6 and this would indicate that in the genome pBC10-0.6 is buried within a 9.7 Kbp HpaI fragment. Whether or not this larger repeated sequence is tandemly repeated or interspersed with other sequences is not yet distinguishable.

The organization of pBC15-1.1 and pBA33-1.8 is more complex. Blots of EcoRI-digested male DNA probed with either of these gives multiple bands with no apparent molecular weight relationship with each other (Fig. 2). The major hybridization signals of pBC15-1.1 is on EcoRI fragments of 4.3 and 3.7 Kbp. Minor hybridization is observed with three fragments of 10.5, 9.4, and 6.6 Kbp; the cognate 1.1 fragment is barely detectable in equivalent exposures. Similar blots probed with pBA33-1.8 yield major hybridization with the 1.8 Kbp cognate EcoRI fragment as well as three larger EcoRI fragments at 10.5, 9.4 and 6.6 Kbp. These latter three appear to be the same fragments hybridizing to pBC15-1.1 noted above, and suggests that the two sequences are located on the same three high molecular weight EcoRI fragments. That they actually do comigrate was verified by a two way blot from agarose gels and demonstration that the high molecular weight bands hybridizing to pBC15-1.1 and pBA33-1.8 are superimposable (not shown).

More definitive support for the interspersion of pBC15-1.1 and pBA33-1.8 on some common EcoRI fragments was obtained during the determination of cross reactivities and interspersion of the three subclonal EcoRI fragments among the five independently isolated genomic phage clones. Collectively these five phage clones represent about 75 Kbp of Y chromosomal DNA. Two of the subcloned male specific EcoRI fragments, pBA33-1.8 and pC15-1.1, were observed to hybridize to a number of other EcoRI fragments from the genomic clones (Fig. 1 and not shown). One of these, from phage C15 hybridized to both pBA33-1.8 and pBC15-1.1. Thus, the arrangement of pBA33-1.8 and pBC15-1.1 is strikingly different from that found for pBC10-0.6. Sequences complementary to parts of each are associated with each other to form a number of different repeat families.

II. Evolutionary Stability of Male Specific Sequences.

All three subclones were used to probe blots of DNA derived from the closely related murid species, Ratus, and its more distant cricetid cousin, Peromyscus. No hybridization was observed with either species (data not shown).

Interesting differences and similarities were observed in comparisons of restriction patterns generated with genomic DNA from laboratory mice and the more closely related Mus poschiavinus (Fig. 2). These species are sufficiently

Figure 4. Presence of pBC10-0.6 homologous sequences in female DNA and their location on the X chromosome. At the right is a Southern blot of EcoRI digested female BALB/c DNA hybridized to replacement synthesized (5 x 10⁸ cpm/μg) ³²P-pBC10-0.6 insert DNA. At the left are tabulated the relative hybridization intensities of globin and two of the pBC10-0.6 reactive female fragments (2.2 and 1.7 Kbp) on blots simultaneously probed with a globin clone and pBC10-0.6.

related so as to be partially interfertile (23). Southern blots of EcoRI digested DNA from both species probed with pBC10-0.6 result in most of the signal being localized to the 0.6 Kbp cognate fragment. Also in both species most of the hybridization of pBC15-1.1 is localized to the two common major EcoRI fragments of 4.3 and 3.7 Kbp. Differences between species were limited to the minor higher molecular weight fragments.

In contrast, most of the hybridization of pBA33-1.8 is to EcoRI fragments clearly distinguishable between the two species. The major 1.8 Kbp cognate fragment present in laboratory mice is missing in *Mus poschiavinus*, whereas converse differences can be seen for a number of other higher molecular weight fragments (Fig. 2).

III. Cross Reactivity of pBC10-0.6, pBC15-1.1, and pBA33-1.8 with Female DNA.

With more sensitive hybridization conditions pBC10-0.6 was observed to hybridize to female DNA (Fig. 4). Four EcoRI fragments are homologous to pBC10-0.6 in female DNA and each of them is distinct from the male specific cognate fragment.

To test the possible location of these sequences on the X chromosome their hybridization intensities in genomic DNA from XX and XO female mice were

Figure 5. Expression of RNA complementary to pBC10-0.6, pBC15-1.1 and pBA33-1.8. Approximately 3 μ g and 0.1 μ g of total cellular RNA from male (M) liver, female (F) liver, testis, male kidney, and male brain were loaded onto nitrocellulose membranes and hybridized with the labeled probes indicated. The filter probed with rDNA was previously probed with pBC10-0.6, and was washed free of label prior to reuse.

compared. Blots prepared from EcoRI digested DNA from three mice of each type were simultaneously probed with pBC10-0.6 and an alpha globin genomic clone as an internal standard. The latter was a 3.2 Kbp SacI fragment provided by Dr. J. Barry Whitney, III (Medical College of Georgia, Augusta). Results of densitometric traces of the hybridization intensity for the 1.7 and 2.2 EcoRI fragments are displayed in Fig. 4. The results are consistent with a two fold greater intensity of both fragments in XX mice by comparison with XO mice. Therefore, the sequences in female DNA that are crossreactive with pBC10-0.6 are located on the X chromosome. Furthermore, a comparison of the hybridization intensity of these sequences with that of alpha-globin would indicate their copy number to be at the unique sequence level.

As noted earlier, pBC15-1.1 and pBA33-1.8 also cross react at the unique sequence level with EcoRI fragments of 3.9 and 3.6 Kbp, respectively, in female DNA. Their chromosomal location is not yet known.

IV. Male Specific Expression of pBC10-0.6, pBC15-1.1, and pBA33-1.8.

Total RNA extracted from various tissues in males and females was tested by dot analysis for hybridization with all three probes and with a cloned ribosomal DNA probe as control (Fig. 5). All three probes hybridized with

Figure 6. Electrophoresis of male liver RNA sequences complementary to pBC10-0.6, pBC15-1.1, and pBA33-1.8. Either 5 μ g of total cellular male liver RNA (T) or 20 μ g of polyA⁺ RNA (A) was fractionated electrophoretically on denaturing agarose gels and Northern blots prepared therefrom. These were probed with the labeled DNAs indicated.

RNA's extracted from a number of male tissues, liver, testis, brain and kidney. In contrast to that from males, female liver RNA hybridized much less or not at all.

To further characterize the nature of the transcripts complementary to each of these probes the polyA⁺ fraction of male liver was isolated, fractionated on glyoxal gels, and analyzed by Northern analysis (Fig. 6). The hybridization of all three probes was predominantly to polyA⁺ molecules larger than the 28S molecules and clustered in two broad bands. The size of each of the two bands was roughly the same for each of the probes. All three probes additionally hybridized to several other species of polyA⁺ molecules ranging in size from larger than the 28S rRNA (5500 nucleotides) to sizes migrating with the dye front (about 75 nucleotides). The hybridization pattern with the rDNA probes displayed the expected 3:1 signal. It is unlikely that this male specific hybridization is to contaminating DNA molecules. Such would not be expected to be enriched by passage through oligo dT columns nor would it be expected to be found in discrete size classes. Furthermore, the signals in the dot hybridization of total RNA were eliminated by prior treatment of the RNA samples with alkali (not shown).

DISCUSSION

We report here a description of three cloned EcoRI genomic fragments whose sequences hybridize almost exclusively with male DNA. The definitive Y chromosomal location of one of the three fragments, pBC15-1.1, was directly demonstrated by in situ hybridization. Although the DNA appeared localized to no specific part of the Y chromosome, the sequences are absent from that part (defined by XX^{SXR} phenotypic males) which is responsible for male sex determination.

These three male specific fragments are reiterated in the range of 100-200 times. Another characteristic in common is that they are rapidly evolving and are virtually undetectable in another murid, the rat. Apart from these common features, they present two striking dissimilarities: the nature and temporal stability of their genomic organization. These became apparent from restriction pattern analyses of inbred mouse strains and the closely related Mus poschiavinus.

One type of sequence organization is that represented by pBC10-0.6, virtually all of whose sequences are embedded within a larger, relatively homogeneous repeat unit at least 9.7 Kbp in size.

A completely distinct type of sequence organization was observed for pBC15-1.1 and pBA33-1.8. Each is found on about 5 or 6 EcoRI families. Three of these families of 6.6 Kbp, 9.4 Kbp, and 10.5 Kbp contain sequences homologous to both pBC15-1.1 and pBA33-1.8. The organization of these latter clones appears similar to the "clustered and scrambled" arrangement described for certain repeat families in Drosophila (24) and chicken (25).

The basis for these two organizational forms is not clear. Possibly the mode by which a family of repeated sequences changes could be dictated by specific features of their sequence. Some repeated sequences may be more apt to undergo rearrangement and scrambling than others. Support for this explanation was observed in blots of EcoRI digested male genomic DNA from laboratory mice and Mus poschiavinus (Figure 2). In such blots probed with pBA33-1.8 most of the repeat families in the two species were different. Whereas, in the opposite extreme, practically all the hybridization to pBC10-0.6 in the two species is to the cognate fragment, indicative of its relative stability.

Our data lead to a further conclusion concerning the sequence composition of the mouse Y chromosome. Among the five genomic phage clones whose selection was based on the presence of repetitive male specific DNA, four were found to hybridize with pBA33-1.8 (Fig. 1). This indicates that pBA33-1.8 sequences are almost always found within 20 Kbp of other male specific repetitive DNA. Based

on this, the copy number estimates for pBA33-1.8 of 130, the insert size in the charon 4A genomic library of about 20 Kbp, and the approximate size of 3×10^4 Kbp for the Y chromosome (9), the proportion of the Y chromosome bearing male specific repeated sequences is about 10%. The remainder of the Y chromosome must be composed of a combination of single copy sequences as well as repeated sequences present throughout the genome.

For all three fragments described here, we found that their male specificity is not absolute. Sequences homologous to each are present in male DNA at about 100 copies/genome, but homologous sequences are also detectable in female DNA at the single copy level and on restriction fragments distinct from those in male DNA. Furthermore, for one of these, pBC10-0.6, the chromosomal location of those sequences in female DNA was found to be the X chromosome.

That there are sequences on the X chromosome showing homology with the ones we found on the Y is not unexpected. The notion of evolutionary relatedness of the X and Y chromosomes was proposed by Ohno (26). He suggested that the X and Y chromosomes are derived from an ancestral pair of homologous chromosomes. This is consistent with the recent demonstration of an homologous X-Y pairing region in which recombination regularly takes place (3,4,5). Further studies of other clones from the human sex chromosomes have also demonstrated considerable sequence homology even outside the pairing region of the X and Y chromosomes (3,27,28,29,30). One of these clones, DXYS1, seems to have been transposed relatively recently from the X chromosome onto the Y (31). Taken together these results indicate that apart from residual ancestral homology between the sex chromosomes, communication between the two is an ongoing process. Studies of the evolutionary history of the male specific sequences described here are likely to bring more understanding of the relationship of the X and Y chromosomes.

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