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Molecular cloning of microdissected lampbrush loop DNA sequences of *Drosophila hydei*

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We microdissected a Y chromosomal lampbrush loop pair from primary spermatocyte nuclei of *Drosophila hydei* and cloned the DNA directly at the microscale. Four of the 12 recombinant DNA clones recovered display *in situ* hybridization to mitotic metaphase Y chromosomes, preferentially in the chromosomal region identified as the origin of the lampbrush loop pair. All clones, however, also hybridize to autosomal and X chromosomal loci in polytene chromosomes. Y chromosomal DNA sequences of *D. hydei* again prove to be members of different families of repeated sequences distributed throughout the genome. These microcloning experiments, which were carried out under very unfavourable experimental conditions (low DNA content of the lampbrush loops in the presence of large amounts of RNA) prove that almost any chromosomal structure detected by light microscopy is directly accessible to molecular cloning experiments by micromethods.

Key words: Drosophila/Y chromosome/microcloning/repetitive DNA/genome structure

Introduction

The molecular analysis of Y chromosomal DNA sequences in *Drosophila* has so far suffered from difficulties in recovering Y chromosome-associated DNA (see Vogt and Hennig, 1983). These difficulties arise from the repetitive nature of probably most of the Y chromosomal DNA sequences, which are not restricted to the Y chromosome but share homologies with other sites throughout the genome (Vogt and Hennig, 1983). This leads not only to difficulties in recovering Y-associated sequences *per se*, but also to difficulties in establishing their true chromosomal origin from the Y chromosome or from other chromosomes.

Our primary interest was focussed on sequences which derive from the large Y chromosomal lampbrush loops in primary spermatocytes of *D. hydei* (Meyer, 1963). Since no Y chromosome-specific probes are available for screening recombinant clone banks of *D. hydei*, we used the microcloning technique developed by Scalenghe *et al.* (1981) to dissect and then directly clone sequences from lampbrush loops of the Y chromosome. Originally this technique was applied to polytene chromosomes which contain several thousand identical copies of the DNA within a single chromosome band. In contrast, the Y chromosome of *Drosophila* does not become polytene. Consequently, diploid cells must be used for microdissection. We therefore tried to clone DNA from distinct loops in spermatocyte nuclei into the λ vector 641 as described before (Scalenghe *et al.*, 1981). Although the amount of DNA recovered in this way was very small, and the conditions in cloning this kind of biological material were very unfavourable, because of the large amounts of ribonucleoprotein associated with the loops, a series of clones was recovered. Their chromosomal distribution was studied by *in situ* hybridization.

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Results

Microdissection of lampbrush loops and molecular cloning

The wild-type spermatocyte nucleus of *D. hydei* is entirely filled with lampbrush loops of the Y chromosome (for review, see Hennig, 1978). In these nuclei, the positions of the autosomes are not precisely predictable and the various loops are not well separated from one another (Yamasaki, 1977, 1981). To overcome this problem, we chose certain X-Y translocations from our strain collection (see Hackstein *et al.*, 1982) and constructed T(X;Y)/O males containing only one or two defined loop pairs in their spermatocyte nuclei (Figure 1a,b). This allowed the recovery of distinct loops without contamination by others. Testes of such males were isolated and squashed between two cover slips (22 x 36 mm). These were placed in liquid nitrogen, and separated with a razor blade. Cover slips with squashes were placed in 70% ethanol for 30 min and then dried.

Micromanipulation was carried out in an oil chamber as described (Scalenghe *et al.*, 1981). The desired loops were scratched out of the nucleus under microscopic control with a glass needle (Figure 1c – e). The material remaining on the tip of the needle was then deposited in a drop of glycerol buffer (0.05 M sodium phosphate buffer, pH 8, 80% glycerol) close to the tissue (Figure 1f). After an adequate number of loops had been collected, the pooled material was transferred with a needle (Figure 1g) to another small cover slip (Figure 1h) and extracted in a microdrop of RNase. DNA was then extracted as described earlier, with only slight modifications. Because of the large amounts of protein in the collected material it was necessary to increase the concentration of SDS to 0.5% (from 0.1%). As a consequence of the increased concentration of SDS, the subsequent phenol extraction for its removal had to be more intense. Two additional phenol extractions were therefore carried out (a total of five). After this step, all following procedures were identical to the published protocol.

Identification and characterization of the cloned DNA sequences

The cloning experiment was carried out with T(X;Y)20/0 males. Their spermatocyte nuclei contain only the lampbrush loop pair 'threads' (Figure 1a). For the cloning experiment, the loops of a total of 1000 nuclei were collected. This amounts to 5 – 50 pg DNA (see Hennig *et al.*, 1974). From an initial yield of 24 prospective clones, only 12 clones displayed a clear plaque morphology on *Escherichia coli* C 3000 in a second plating. In eight (dhMiF1, 2, 4, 5, 8, 9R, 10, 12) out of the 12 clones separate insert bands could be detected in

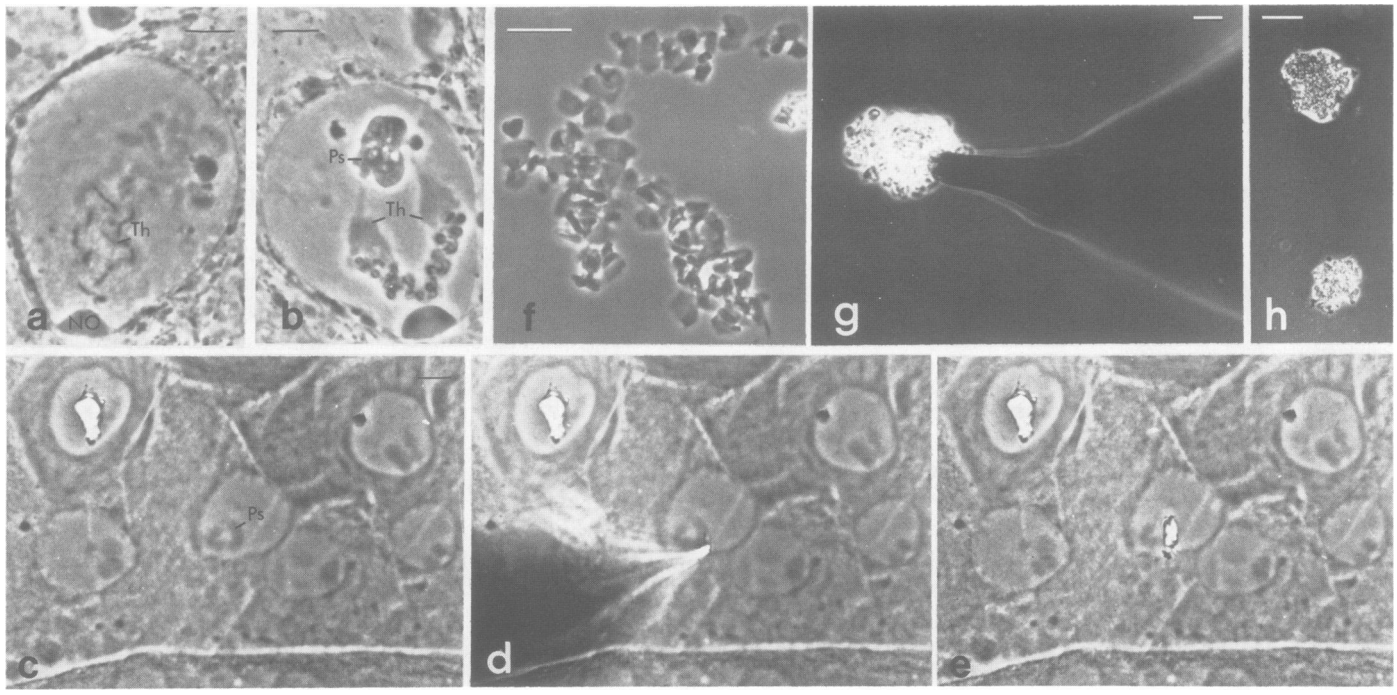

Fig. 1. Microdissection of Y chromosomal lampbrush loops from primary spermatocytes of *D. hydei*. **(a)** Spermatocyte nucleus of a T(X;Y)20/0 testis. Only the lampbrush loop pair 'threads' is present. Such nuclei were used for the cloning experiment described in this paper. **(b)** Spermatocyte nucleus of a T(X;Y)37/0 testis. The photographic demonstration **(c–e)** of the microdissection procedure uses this type of spermatocyte since the structure of the loop 'pseudonucleolus' is more convenient for demonstrative purposes. Th: threads, Ps: pseudonucleolus, NO: nucleolus. **(c)** spermatocyte nuclei before dissection. **(d)** The procedure of dissection with a glass needle. **(e)** Nucleus after dissection. **(f)** Dissected pieces of material are collected in a drop of buffer. **(g)** After collecting sufficient material (~1000 loops), the pieces are transferred to the final incubation medium on the tip of a needle. **(h)** Batches of material after transfer to the incubation buffer. The various droplets seen contain 0.5–1.5 nl buffer and 300–800 pieces of collected material. Bar is 5 μm in **a** and **b**, and 10 μm in **c–h**.

Table I. Characterization of insert sequences

dhMiF	1	2	3	4	5	6	7	8	9R	10	11	12
Insert length (kb)	3.7	3.1 1.7	?	7.5	2.6 1.7	9.3[a]	9.3[a]	2.4 1.9 0.6	6.2	2.9	?	2.6 1.2
In situ hybridized												
metaphases	Y X	Y X	–	–	–	–	–	Y X	–	Y X	–	–
polytene chromosomes	3-63A kineto-chore	X-1 X-2A 2-21A 2-31A 3-49 3-59A 3-69 4-71D 4-72A 4-89B/C 5-98A kineto-chores	–	–	–	–	–	X-1 X-2A 4-72A	NO	X-11B/D	–	–

[a]Inclusive short arm of the vector

restriction gels after *Eco*RI digestion (Table I). In two additional clones (dhMiF 6, 7), one of the *Eco*RI restriction sites used for cloning had disappeared. Since these two clones did not hybridize with DNA of *D. hydei*, they were not further investigated. The insert length recovered in the eight clones with defined inserts varied between 2.9 kb and 7.5 kb (Table I).

The chromosomal origin of the cloned DNA fragments

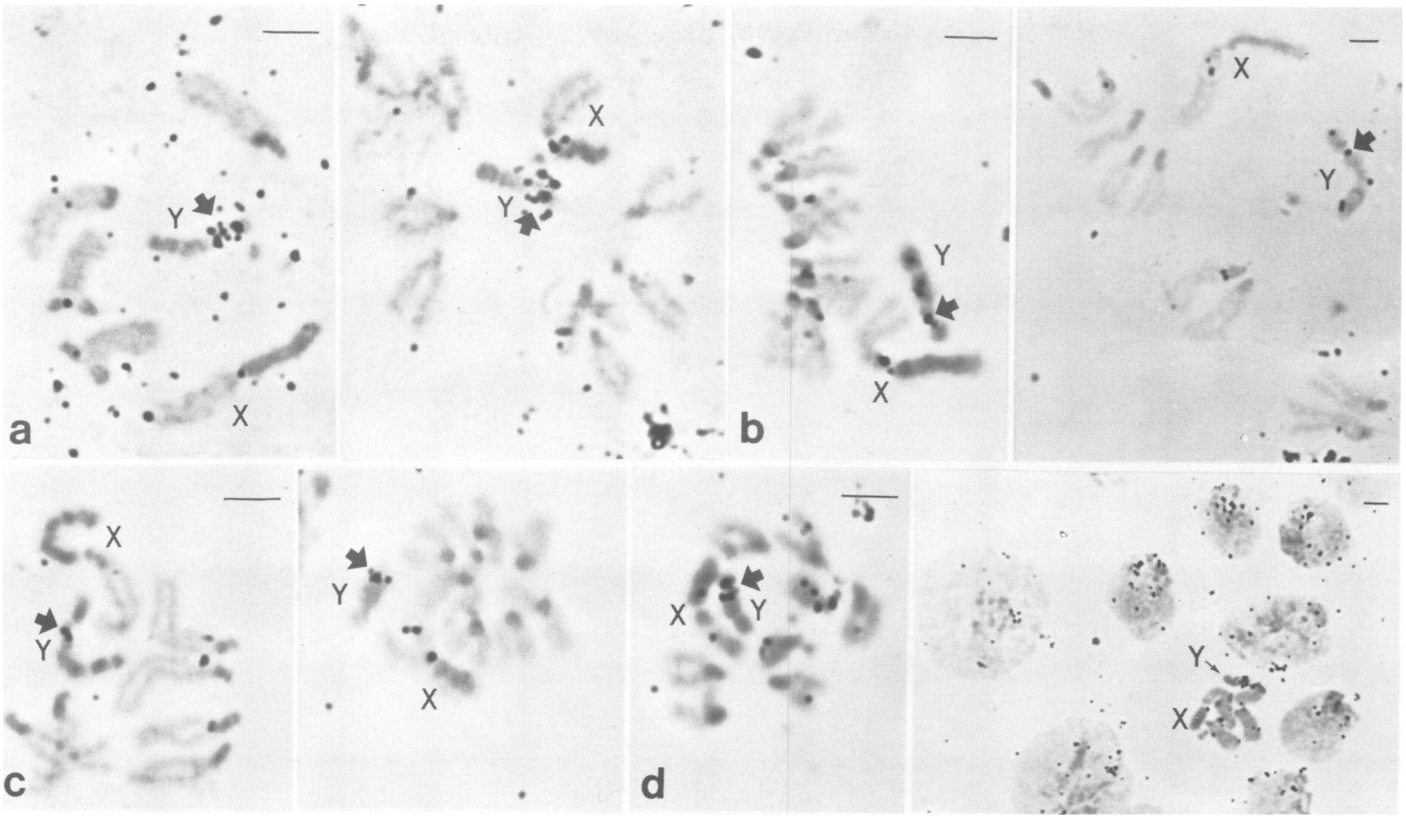

Fig. 2. *In situ* hybridization on neuroblast metaphase chromosomes. The thick arrows indicate the position in the Y chromosome where loci A and B are located. For each clone, two representative metaphase plates are shown. **(a)** dhMiF2, **(b)** dhMiF1, **(c)** dhMiF8, **(d)** dhMiF10 (note distribution of label in the interphase nuclei in the second photograph). Bar is 5 μm.

was shown by hybridization *in situ* of tritiated cRNA complementary to the cloned inserts to mitotic metaphase chromosomes (Figure 2) or to polytene chromosomes from salivary glands (Figure 3). Significant hybridization was obtained with five clones (dhMiF1, 2, 8, 9R and 10). The most obvious label in mitotic chromosomes was found in the Y chromosome close to the end of the long arm (thick arrows in Figure 2). In addition, other sites in the Y chromosome are labeled in hybridization experiments using the clones dhMi2 and 8. Besides these Y chromosomal locations, the X chromosome is often labeled in a small region close to its kinetochore. Some autosomal label is occasionally seen. However, since the autosomes cannot be recognized individually in mitotic metaphases, it is difficult to ascertain the labeling of distinct autosomes.

Information on autosomal locations of the cloned DNA sequences can more easily be obtained with polytene chromosomes (Figure 3). The same clones displaying *in situ* hybridization with mitotic metaphase chromosomes also react with polytene chromosomes. In all cases, one to a few bands in euchromatic regions of the autosomes are labeled. In addition, label is often seen in the kinetochore region of the X chromosome as was to be expected from the labeling pattern in mitotic metaphases. Clone dhMiF9R hybridizes strongly to the nucleolus (Figure 3). It also hybridizes with rRNA in a DNA blot and, therefore, represents a region of the rDNA. From the isolation procedure it was to be expected that rDNA clones could be recovered since the 'threads' are immediately adjacent to the nucleolus (Figure 1a). rRNA genes might occasionally be closely associated with Y chromosomal lampbrush loops (Grond *et al.*, 1983). The cloning of rDNA sequences may therefore serve as an internal control for the cloning experiment. Taken together, the *in situ* hybridization experiments identified the cloned DNA sequences as members of families of repeated sequences with copies both in the Y chromosome and in other chromosomes.

The Y chromosomal origin of the cloned sequences should also become evident in DNA blots of genomic DNA from males or females after restriction and subsequent electrophoresis in agarose gels (Southern, 1975). When such experiments were carried out rather unexpected results were obtained. With none of the cloned sequences was a significant qualitative or quantitative difference in hybridization to DNA from males or females detected (Figure 4), irrespective of the restriction enzymes used. Only after subcloning fragments of the DNA inserts could some male-specific restriction fragments be identified in genomic blots. An example is shown in Figure 5. This proves that the cloned DNA sequences are linked to Y-specific restriction fragments. Also in these blots the majority of restriction fragments do not differ in size or intensity between the DNA from males or females. This indicates that either the number of copies of these sequences is approximately the same in both the X and the Y (cf., Figure 2b) or that the total number of copies in the Y chromosome is small compared with the rest of the genome.

A comparison of the hybridization patterns of genomic DNA digests from two related species, *D. neohydei* and *D. eohydei* (Figure 4), also shows differences between males and females, indicating a Y chromosomal location of sequences homologous to the cloned *hydei* sequences.

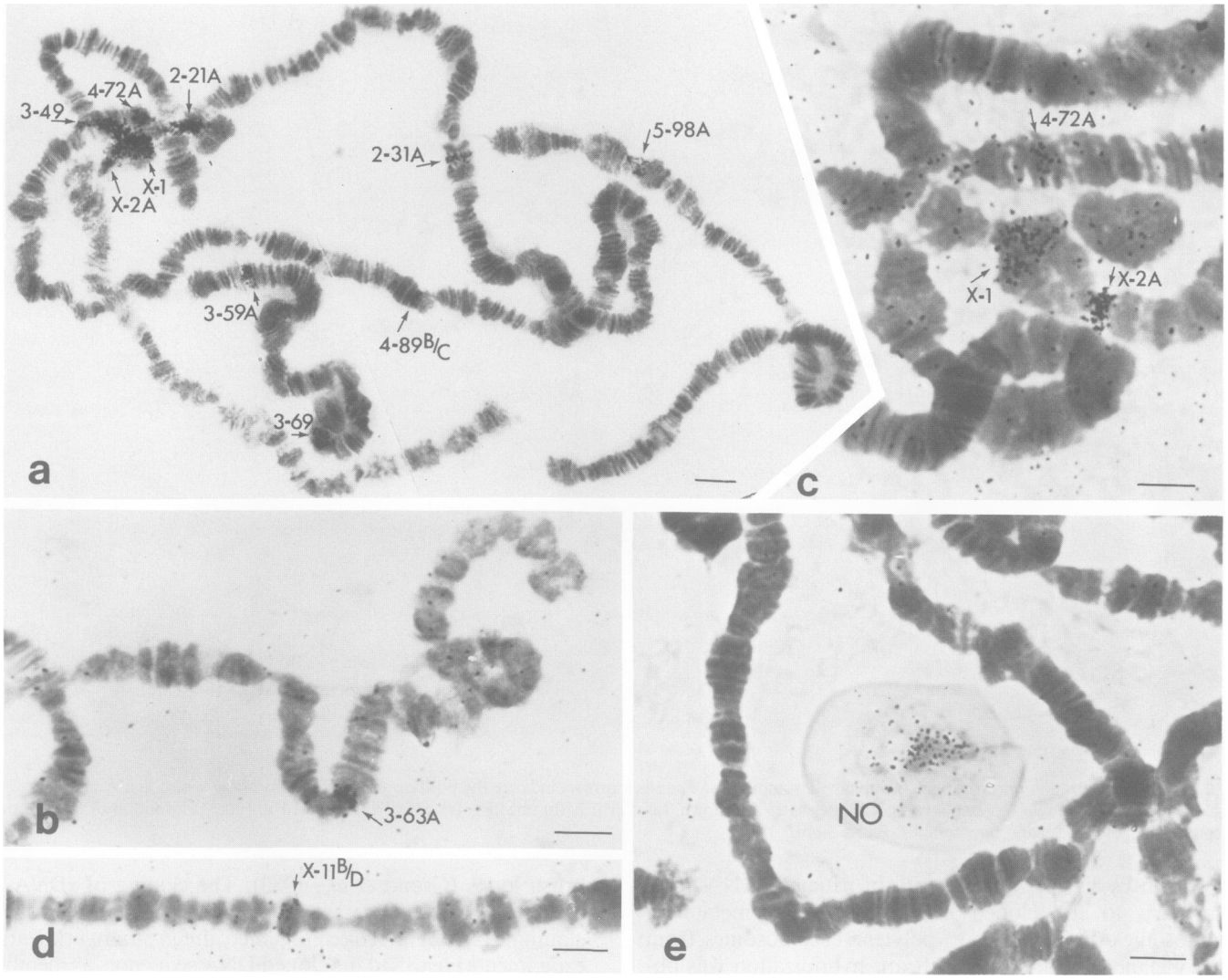

Fig. 3. *In situ* hybridization on polytene chromosomes. **(a)** dhMiF2, **(b)** dhMiF1, **(c)** dhMiF8, **(d)** dhMiF9R, **(e)** dhMiF10. Bar is 10 μm.

In conclusion, our data indicate that extensive homologies exist between the DNA in the Y chromosome and the rest of the genome. Moreover, a high degree of sequence conservation in different genomic positons and during evolution was found, and the majority of the copies must be located in the autosomes.

Discussion

The experiments reported in this paper demonstrate that microcloning techniques can also be used for non-polytene chromosome regions, even when the DNA concentration is very low.

The impact of the present experimental approach is evident from the data provided in this and other papers (Vogt *et al.*, 1982). It has been shown that Y chromosomal sequences in general are shared by many different loci throughout the genome. Therefore, the recovery of copies located in the Y chromosome by conventional cloning approaches is difficult (Vogt *et al.*, 1982; Lifschytz *et al.*, 1983; Vogt and Hennig, 1983). In addition, it may be difficult to establish that the cloned sequences truly originate from the Y chromosome since the copies in different regions of the genome display little sequence divergence. Moreover, recovery of distinct Y chromosomal sequences by 'chromosome walking' is unlikely to succeed because of the repetitive nature of most of the Y chromosomal DNA sequences.

The relationship of the microclones obtained and the lampbrush loops used for microdissection has not yet been studied in detail. However, the *in situ* hybridization experiments prove that the cloned sequences hybridize to the region of the Y chromosome from where they should originate, i.e., close to the end of the long arm. In Figure 2 it can be seen that the label is always associated with a less strongly staining region close to a more strongly staining chromosome region at the end of the long arm. This localization of the cloned DNA sequences is in agreement with the chromosomal location of locus A, which is defined as the complementation group associated with the 'threads', and has now also been confirmed by cytogenetic analysis. Loci A – C (Hackstein *et al.*, 1982) have been assigned (Bonaccorsi, Hackstein and Hennig, in preparation) to the first chromosome region not staining with Hoechst 33 258 (cf., Bonaccorsi *et al.*, 1981).

The *in situ* hybridization experiments described here further confirm and extend the information on the sequence composition of the Y chromosome and its relationship to other chromosomes. From studies of cloned Y chromosomal DNA sequences obtained by conventional methods (Vogt and

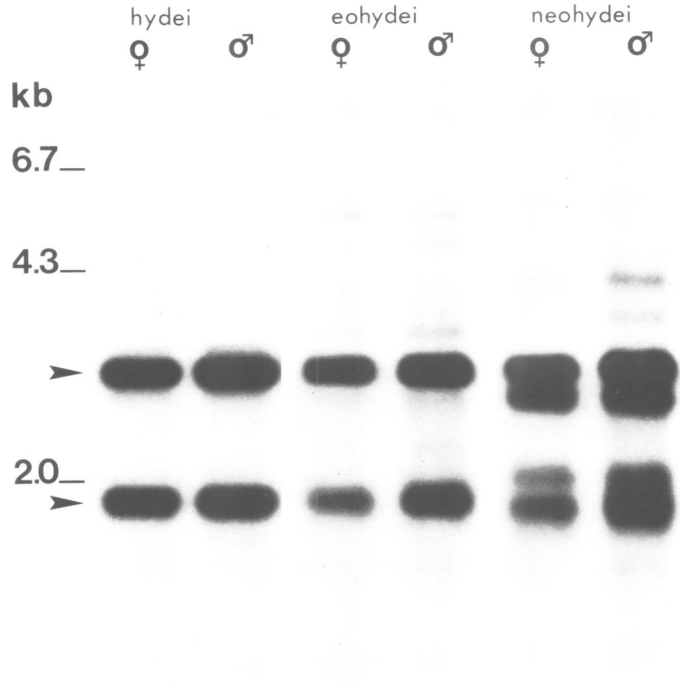

Fig. 4. Blots of *Eco*RI totally digested genomic DNA from *D. hydei*, *D. eohydei* and *D. neohydei* males and females. For hybridization, nick-translated DNA of dhMiF2 was used as a probe. Marker was *Hind*III digested λ DNA (at the left). The arrowheads indicate the position of the bands representing the insert of the clone. From partial digests we deduced that they originate from a genomic sequence of 4.8 kb (insert size) which carries one internal *Eco*RI site (not shown). In *D. hydei* no differences in hybridization of DNA from males or females can be detected. In the other species, however, male-associated fragments become apparent. Incubation was in 4 x SET (68°C), washing in 0.1 x SSC (68°C). An overexposed autoradiogram is shown to display minor bands.

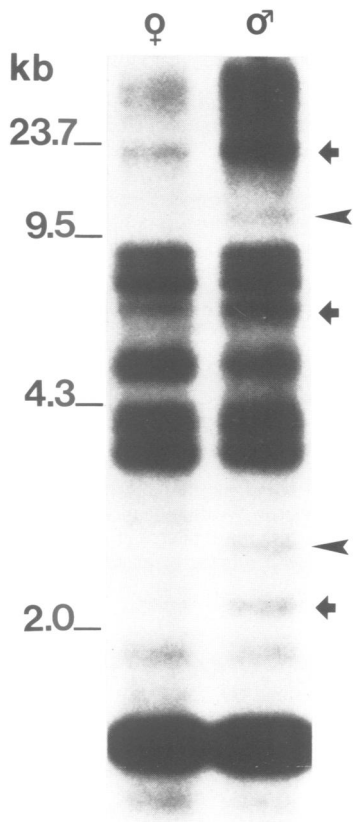

Fig. 5. Blots of *Pvu*II digested genomic DNA of *D. hydei* females and males. For hybridization, nick-translated DNA of a subcloned 3.2-kb *Eco*RI fragment of dhMiF2 (see Figure 4, first lane) was used as a probe. Marker was *Hind*III digested λ DNA (at the left). The arrowheads indicate the positions of male-specific (i.e., Y chromosome-specific) restriction fragments, the arrows indicate the positions of restriction fragments preferentially located in the Y chromosome. Incubation was in 4 x SET (68°C), washing in 0.1 x SSC (68°C).

Hennig, 1983), we deduced that most of the Y chromosomal DNA sequences of *D. hydei* are represented elsewhere in the genome. This also holds true for the sequences obtained by microcloning. In fact, these sequences cannot be detected as Y-associated with the methods used by Lifschytz (1979) and Vogt and Hennig (1983). There appears to be no particular preference for distinct chromosomes or chromosomal regions since sequence homologies have been found throughout the genome. The DNA blots indicate, furthermore, that the majority of the copies homologous to the cloned DNA sequences must reside outside the Y chromosome, as otherwise quantitative differences in the hybridization patterns of DNA from males or females would be expected. Although different accessibility of the chromosomal DNA sequences for *in situ* hybridization cannot be excluded, the hybridization signals in the Y chromosome obtained after *in situ* hybridization suggests a clustering of the Y chromosomal copies. Autosomal and X chromosomal copies must be located in a more dispersed pattern. This interpretation is supported by the *in situ* hybridization patterns in polytene chromosomes.

The results of the DNA blots indicate a high degree of sequence conservation in the different genomic locations. This, together with the dispersed location, is reminiscent of 'nomadic' DNA sequences. However, the sequence distribution of the Y-associated sequences is not compatible with the general pattern of 'nomadic' moderately repetitive sequences [such as *copia*, the *FB* family of *mdg* elements etc. (Young and Schwartz, 1981; Truett *et al.*, 1981; Ananiev *et al.*, 1978)]. In most cases we found only few labeled chromosome regions in polytene chromosomes. The sequences often reside in kinetochore-associated heterochromatin. Moreover, *copia*-like sequences appear to be rather species specific (cf., Dowsett and Young, 1982; Dowsett, 1983). This is not true for the Y chromosomal sequences of *D. hydei* considered in this paper (see Figure 4). Studies of Y chromosomal DNA sequences in other organisms revealed features similar to those of *D. hydei* (e.g. Nallascht, 1983, Kunkel and Smith, 1982).

The observations reported here reveal another feature of Y chromosomal sequences: homologous sequences may occur in different positions along the Y chromosome. This is particularly evident for clone dhMiF2, which is found in at least three different regions throughout the chromosome. Since the clones must be derived from a chromosome region contained in one of the lampbrush loops, it will be of interest to see whether the homologous sites are correlated with other Y chromosomal genes. Similar cross-homologies have been found for clones derived from the short arm of the Y chromosome of *D. hydei* (Vogt and Hennig, 1983).

Materials and methods

Drosophila strains

For microcloning Y chromosomal DNA sequences, T(X;Y)/0 males of the translocation T(X:Y)20 (Hackstein and Hennig, 1982) have been used, which

carry only loci A and B in the translocation fragment of the Y chromosome. Cytologically only the loop pair 'threads' is present. *In situ* hybridization experiments were done with wild-type males of *D. hydei* (Tübingen).

Microcloning

The microcloning was carried out as described by Scalenghe *et al.* (1981) with the following modifications. The dissected loops were digested in 2 nl buffer (0.01 M Tris, pH 8, 10 mM NaCl) with 100 μg/ml pancreatic RNase (preheated) for 30 min at room temperature. Then SDS was added to a final concentration of 0.5% and pronase K to a final concentration of 0.5 mg/ml. Incubation was 60 min at 37°C in a humid chamber.

In situ hybridization

In situ hybridization was carried out as described (Hennig *et al.*, 1982).

DNA blots

DNA blotting, nick-translation and hybridization was carried out as described (Vogt and Hennig, 1983).

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