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Structure and function of *Y* chromosomal DNA

II. Analysis of lampbrush loop associated transcripts in nuclei of primary spermatocytes of *Drosophila hydei* by in situ hybridization using asymmetric RNA probes of four different families of repetitive DNA

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Abstract. pSP64/65 subclones of four different families of repetitive sequences on the *Y* chromosome of *Drosophila hydei* were used for in vitro synthesis of labelled RNA. Pairs of RNA probes of opposite strand polarity were employed to analyse RNAs transcribed on, or associated with, various *Y* chromosomal lampbrush loops in nuclei of primary spermatocytes of *D. hydei*. The results of RNA filter analysis and in situ hybridization experiments can be generalized as follows: (1) *Y*-specific transcripts are heterogeneous in length and are synthesized on lampbrush loops. (2) Transcription of tandemly repeated sequences is usually strand specific. (3) Members of the same sequence family can be found in transcripts from different lampbrush loops. (4) Transcripts not coded by the *Y* chromosome are accumulated on different subregions of *Y* chromosomal lampbrush loops.

Introduction

Primary spermatocytes of *Drosophila hydei* provide a fascinating system for the study of several unsolved problems of molecular genetics such as the organization and function of genes embedded in heterochromatin (for a recent review see Pimpinelli et al. 1986) and the fate of primary transcripts in germ line cells (Dearsly et al. 1985). The nuclei of these *D. hydei* cells are large (30–50 μm) and contain a number of characteristic chromatin structures (Fig. 1 A). Each particular structure is associated with one of a small number of lampbrush loops designated according to their morphology as nooses (Ns), clubs (Cl), tubular ribbons (Tr), pseudonucleolus (Ps) and threads (Th) (Fig. 1 A, B) which unfold from the *Y* chromosome in the growing spermatocyte. A possible functional significance of these structures has been deduced from mutant strains in which a clear correlation between the occurrence of one of these structures and the activity of (at least) one of the five so-called fertility factors exists (Hess 1967). These fertility genes are of unusual length (1000–4000 kb) and are located on different regions of the heterochromatic *Y* chromosome (Hackstein et al. 1982) in close spatial contact with various repetitive sequences distributed along the chromosome. Recently some of these repetitive sequences have been cloned and characterized by sequence analysis (Lifschytz 1979; Hennig et al. 1983; Vogt et al. 1982; Lifschytz et al. 1983; Vogt and Hennig 1983, 1986a, b; Awgulewitsch and Bünemann 1986; Wlaschek et al. 1988).

Although the vast majority of these sequences have been identified within spermatocyte specific transcripts originating on particular lampbrush loops, definite functions in the course of spermatogenesis have not yet been ascribed to any of these repetitive sequences. Since all lampbrush loop specific transcripts are of enormous size and very heterogeneous in length (Glätzer and Meyer 1981; Grond et al. 1983, 1984; de Loos et al. 1984) their molecular analysis is extremely difficult. In spite of the many efforts which have been made to isolate *Y* chromosomal gene products neither RNAs of defined length nor proteins coded for by this chromosome have been detected (for recent review see Hennig 1985). It is therefore uncertain whether any 'classical genes' are present on the *Y* chromosome. Consequently, all recent models for the functions of fertility genes have been based on the few details which are known about the molecular components of the spectacular lampbrush loops. The detection of specific proteins associated with different loops (Glätzer 1984; Hulsebos et al. 1984; Glätzer and Kloetzel 1985, 1986) has been interpreted as the result of specific complexes formed between transcripts and several proteins accumulated and stored as ribonucleoproteins (RNP) for use during later sperm development. Alternatively, it has been proposed that the RNP structures formed on particular lampbrush loops are responsible for the 'proper compartmentalization of gene activity and sequestration of gene products for postmeiotic differentiation' (Hareven et al. 1986).

Recently the data on the organization and function of the fertility genes of *D. hydei* have been increased substantially by the isolation and characterization of four additional families of *Y* chromosomal repetitive DNA (Awgulewitsch et al. 1986; Wlaschek et al. 1988). The different families have been named Y_SI and Y_LI–Y_LIII according to their localization on the short or long arm of the *Y* chromosome, respectively (Fig. 1 C). All families are organized in clusters and consist of tandemly arranged family specific degenerated repeat units. All can be detected as components of spermatocyte specific RNAs of heterogeneous size (Bünemann, unpublished). This length heterogeneity is a characteristic feature of all lampbrush loop specific transcripts in *D. hydei* described so far (Vogt et al. 1982; Lifschytz et al. 1983).

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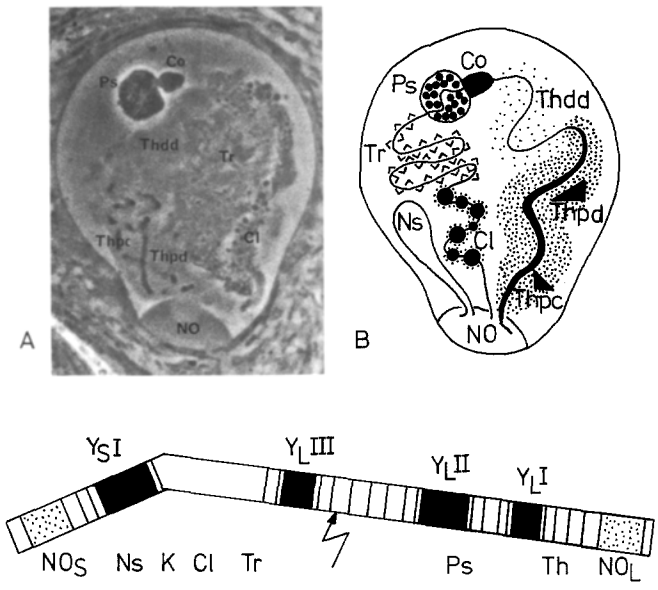


Fig. 1 A–C. Diagrammatic key to the nuclear structures in primary spermatocytes of *Drosophila hydei* and their correlation with various *Y* chromosomal markers. **A** A phase contrast photomicrograph depicting a nucleus of a primary spermatocyte cell. Several characteristic *Y* chromosomal lampbrush loops are visible: clubs (*Cl*), tubular ribbons (*Tr*), pseudonucleolus (*Ps*), cones (*Co*) and threads (*Th*) subdivided in structurally different regions 'distal diffuse' (*dd*), 'proximal diffuse' (*pd*) and 'proximal compact' (*pc*). **B** Schematic diagram of **A**, modified according to Glätzer (1984). The lampbrush loops nooses (*Ns*) not visible in **A** are shown as well as the nucleolar organizer (*NO*). **C** A schematic map of the *Y* chromosome correlating the fertility genes (*Ns, Cl, Tr, Ps, Th*) with several clusters of repetitive sequence families (Y_SI, Y_LI–Y_LIII). *Dark areas* indicate the approximate extension of these clusters. Regions with small numbers of these repetitive sequences are *hatched.* The kinetochore (*K*) and the nucleolar organizers on both ends of the chromosome (*NO*L and *NO*s) are indicated. A *flash symbol* marks the approximate position of the breakage event which gave rise to the translocation chromosomes containing the *Y* chromosomal halves Y^{NsClTr} and Y^{PsTh} used throughout the in situ hybridization experiments

When the Y_SI (Y_SIa) and Y_LI–Y_LIII sequences are compared with the available data on other *Y* chromosomal clones only the Y_SI and Y_SIa clones are related to published sequences (Wlaschek et al. 1988; Vogt et al. 1982; Lifschytz et al. 1983; Lifschytz and Hareven 1985; Vogt and Hennig 1983, 1986a, b). All sequences related to the Y_SI and Y_SIa subfamilies are situated in the short arm of the *Y* chromosome and are found in transcripts of the lampbrush loop Ns (Fig. 1C). The other families, Y_LI–Y_LIII, are localized in the long arm but do not show homology with other sequences thought to originate form this arm of the *Y* (Wlaschek et al. 1988; Lifschytz et al. 1983; Hennig et al. 1983; Huijser and Hennig 1987; Hareven et al. 1986). Taken together, the total number of cloned *Y* chromosomal sequence families exceeds the number of discernible loops in the spermatocyte nuclei of *D. hydei.* Therefore the hypothesis that each loop only contains a single specific family of repetitive DNA (Lifschytz et al. 1983) has been questioned. It is clearly essential that the cytological localization of transcripts containing the newly isolated sequences must be determined before the RNP structures associated with various lampbrush loops can be specified. Naturally, experiments to localize these transcripts are critically dependent upon the sensitivity and specificity of hybridization conditions. We therefore used asymmetric ^{3}H-labelled RNAs (Cox et al. 1984) transcribed in vitro by SP6 polymerase from pSP64/65 subclones according to Melton et al. (1984) instead of the strand unspecific RNA and DNA samples utilized by Vogt et al. (1982) and Lifschytz et al. (1983). By using spermatocytes of various genotypes and comparing hybridizations performed with transcripts from one strand of a cloned sequence with hybridizations performed with transcripts from the complementary strand the conclusions drawn from earlier experiments about transcription of *Y* chromosomal sequences could be confirmed. Moreover, these particular findings could be extended, by demonstrating the association of various RNA species not coded by the *Y* chromosome with different parts of *Y* chromosomal lampbrush loops.

Materials and methods

Drosophila stocks. All stocks were taken from the *D. hydei* stock collection of O. Hess (Düsseldorf). The combination stocks KOM 697/16: $XX/Y^{Ns.ClTr}$; $A/A \times XY^{PsTh}/Y^{Ns.ClTr}$; *A/A* and KOM 290/2: XX/Y^{PsTh}; $A/A \times Y^{Ns.ClTr}$ $X/A.Y^{PsTh}$; *A/A* were used for the production of spermatocyte nuclei of type $X/Y^{Ns.ClTr}$ and $X/A.Y^{PsTh}$, respectively (Hess 1970). The two fragments of the *Y* chromosome were the result of a single X ray induced breakage event indicated by the flash symbol in Figure 1C. Both fragments are complementary with respect to full fertility and carry the loop-forming sites Ns, Cl, Tr and Ps, Th, respectively. *X/O* males were produced according to Beck (1976).

Preparation of D. hydei RNA. Larval testes anlagen were isolated by a modification of the method of Boyd et al. (1968) by squashing late third instar larvae. The testes anlagen were isolated with tweezers under a stereomicroscope from the final sediment in *Drosophila* Ringer (10 mM KCl, 60 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2) and collected by centrifugation at 1,000 rpm for 2 min (Kloetzel et al. 1981). After removal of the supernatant the testes anlagen were transferred into a 1 ml Dounce homogenizer and disrupted in 4 M guanidinium thiocyanate. Isolation of RNA was performed by centrifugation through a 5.7 M CsCl step gradient according to Chirgwin et al. (1979).

Synthesis of asymmetric RNA probes. Pairs of complementary plasmids, containing the inserted DNA in the opposite orientation with respect to the SP6 promoter (Wlaschek et al. 1988), were linearized by restriction endonuclease digestion at the SmaI or HindIII site in their polylinker region, purified by phenol/chloroform/ethanol treatment and dissolved in sterile diethylpyrocarbonate-treated water. Asymmetric RNA transcript probes (9×10^7 dpm/µg) were synthesized from pSP64/65 DNA templates in 25 µl reaction mixtures containing 30 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine, 500 µM each ATP, CTP and GTP, 47 mM ^{3}H-UTP (53 Ci/mmol, 1 Ci = 37 GBq; Amersham), ribonuclease inhibitor (1 U/µl), 1 µg DNA template and 4.5 U SP6 polymerase, essentially as described by Melton et al. (1984). (For RNA filter analysis ^{3}H-UTP was replaced by ^{32}P-UTP, 400–600 Ci/mmol.) Transcription at 40° C was stopped

after 80 min. Typically, transcription from the various templates resulted in 15%–50% incorporation of the labelled precursor. After digestion of template DNA with RNase-free DNase (0.1 μg/μl) the transcripts were purified by phenol/chloroform/ethanol treatment and dissolved in 25 μl sterile, diethylpyrocarbonate-treated water and stored at −20° C.

RNA filter hybridization. To separate RNA for blotting 2.2 M formaldehyde/1.5% agarose gels were used (Lehrach et al. 1977). The gel buffer used was 20 mM MOPS (3-[N-morpholino] propanesulphonic acid), 5 mM sodium acetate, 1 mM disodium EDTA, pH 7.0. Usually 2 μg of total RNA of larval testes anlagen were loaded onto each lane (Fig. 3). RNA was transferred from gels to nitrocellulose using the procedure of Thomas (1980). Hybridization to filters was performed in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate), 5 × Denhardt's [0.1% Ficoll, 0.1% polyvinylpyrolidone (PVP), 0.1% BSA; Denhardt 1966], 50 mM sodium phosphate, pH 6.0 and 0.2 mg/ml sheared salmon sperm DNA for 24–48 h at 42° C. Labelled RNA probes (5×10^8 cpm/μg) were prepared by in vitro transcription of pSP64/65 subclones with SP6 polymerase as described above. The filters were washed several times for 15 min in 2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS at room temperature and 42° C, respectively. For autoradiography Kodak X-Omat AR film and 2 Dupont Lightning-Plus screens were used and the films exposed at −70° C.

Preparation of spermatocyte nuclei for in situ hybridization. Testes from freshly emerged adult males were dissected in *Drosophila* Ringer, transferred into a drop of the same solution on a clean slide, squashed under a siliconized coverslip and frozen in liquid nitrogen. After removal of the coverslip the preparations were fixed first in 96% ethanol for 2 min and then 5 min in 3.7% unbuffered formaldehyde solution (Glätzer 1984). Slides were washed successively in 0.1 × SSC (2 × 10 min), 30% ethanol (2 × 5 min), 50% ethanol (1 × 5 min), 70% ethanol (1 × 5 min), 96% ethanol (2 × 10 min) and air dried.

In situ hybridization. ^{3}H-RNA probes (30,000 cpm/slide) and carrier (500 μg/ml yeast tRNA) were boiled for 3 min in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, quick-cooled in ice water and mixed with other components to give the following final concentrations: 50% formamide (deionized by mixed bed ion exchanger), STE (0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA), 1 × Denhardt's (0.02% each BSA, Ficoll, PVP) and 5% dextran sulphate, essentially according to Cox et al. (1984). Hybridization mixtures (12–15 μl) were applied to the slides with fixed spermatocytes, covered with siliconized coverslips and kept in a tightly sealed moist chamber at 45° C for 12–18 h. Following hybridization the coverslips were removed by dipping into 2 × SSC. Non-specifically bound nucleic acid probes were removed by succeeding washes in 2 × SSC and 0.1 × SSC (2 l total volume each). After final dehydration in 30% ethanol (2 × 5 min), 50% ethanol (1 × 5 min), 70% ethanol (1 × 5 min) and 96% ethanol (2 × 10 min) the slides were air dried, dipped into NTB-2 (Kodak) nuclear track emulsion and stored in light-tight plastic boxes for several days at 15°–20° C. Development was carried out with Kodak D-19 developer as recommended by the manufacturer.

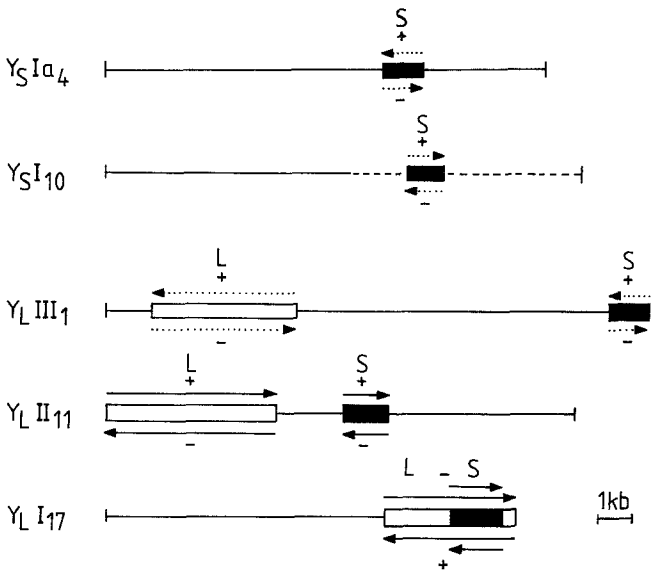


Fig. 2. Survey of the family specific subclones used for preparation of asymmetric (+) and (−) in vitro transcripts. The maps for the various phages ($Y_S Ia_4 - Y_L I_{17}$) are reduced versions of those in Figure 2 of the accompanying paper (Wlaschek et al. 1988) and show the family specific subclones employed for the hybridization experiments. The short and long subclones of each particular sequence family are designated as *filled bars* (*S*) and *open bars* (*L*), respectively. *Broken arrows* represent transcripts of subclones with unknown orientation within the phage map. The designations (+) and (−) were chosen arbitrarily before any results of hybridization experiments were known. Therefore (+) is not systematically correlated with positive hybridization to the transcripts of lampbrush loops

Results

Preparation and characterization of pSP64 subclones of Y chromosomal repetitive sequences

Twenty-one different clones of *Y* chromosomal DNA in EMBL3 phages, picked and assorted according to four characteristic hybridization patterns on blots of AluI-digested genomic DNA of males and females (Awgulewitsch and Bünemann 1986), provided the source material upon which this study is based. The four families have been named $Y_S I$ ($Y_S Ia$) and $Y_L I$–$Y_L III$ according to their location on the short and long arm of the *Y* chromosome, respectively (Wlaschek et al. 1988). Each family comprises its own characteristic repeat unit ranging in size from a few to several hundred base pairs. We selected representative phage clones from each family according to the convenience with which they could be subcloned in pSP64/65. Hence, phage 1 ($Y_L III$), phage 11 ($Y_L II$), phage 17 ($Y_L I$) and phage 10 ($Y_S I$) were chosen because their DNA inserts could be cut by SalI into several fragments of different size (see Fig. 2 in Wlaschek et al. 1988). Only the insert of phage 4 ($Y_S Ia$), representative of a subfamily of $Y_S I$, was insensitive to SalI digestion and in this case EcoRI was used for subcloning. All fragments of interest (filled and open bars in Fig. 2) were cloned in both orientations in pSP64 or 65 to allow the in vitro synthesis of complementary (+) and (−) transcripts by SP6 polymerase (symbolized by arrows of opposite direction in Fig. 2). In the course of this work we used two series of subclones shown as filled or open bars in Figure 2. The filled bars represent

short (S) and completely sequenced clones whereas open bars designate long (L) clones with sequence homology to the short ones. Further details of sequence analysis are described in the accompanying paper (Wlaschek et al. 1988). For reasons of clarity we use abbreviated designations for the various subclones of Figure 2. Number suffixes appended to the family name, e.g. Y_LIII_1, refer to the phage number. Additional (S) and (L) appendices discriminate short and long clones, respectively. Since each particular phage insert appears to be constructed essentially of shorter family specific repeats (Wlaschek et al. 1988) each (S) and (L) subclone of one particular sequence family is expected to contain several copies of these family specific repeats.

Analysis of Y chromosomal transcripts by strand-specific RNA samples

All previous experiments with labelled RNA and DNA probes of various repetitive sequences of the *Y* chromosome have confirmed the occurrence of those sequences in spermatocyte specific transcripts of heterogeneous length (Vogt et al. 1982; Lifschytz et al. 1983; Lifschytz and Hareven 1985). Recently it has been reported that transcription of repetitive sequences occurs in a strand-specific manner (Lifschytz and Hareven 1985). If the same principle of transcription could be verified for each of our four sequence families an identical orientation of all repetitive elements of a particular family, at least within a cluster of transcribed sequences, could be assumed as the general buidling scheme for repetitive sequences on the *Y* chromosome, very similar to the organization of rRNA genes within nucleolar organizers.

The analysis of *Y* chromosomal RNA species using strand-specific (+) and (−) in vitro transcripts of all four sequence families is illustrated in Figure 3A. Each filter was prepared with identical amounts of total RNA from larval testes anlagen of *X/Y* and *X/O* males and was hybridized with labelled (+) and (−) in vitro transcripts of the same specific activity under otherwise identical reaction conditions. The relative amounts of each reacting RNA species can therefore be estimated approximately from the strength of hybridization signals in Figure 3A. Two series of hybridizations are shown in the figure, the upper one corresponds mainly to in vitro transcripts from the (L) clones, the lower one to that of the (S) ones (see Fig. 2). In the case of (S*) three different short clones were tested within separate experiments instead of a single long one. When both sets of experiments are compared several different types of RNA species crossreacting with the in vitro probes can be distinguished within total RNA of larval testes anlagen (an organ filled with growing spermatocytes) of *D. hydei*. (1) RNAs that are heterogeneous in size and transcribed exclusively from the *Y* chromosome (the crossreacting RNAs are restricted to the *X/Y* lanes): $Y_LI_{17}^+$ (L) and (S), $Y_LII_{11}^+$ (L) and (S), $Y_LII_{11}^-$ (L), $Y_SI_{10}^-$ (S*) and $Y_SIa_4^-$ (S*). (2) RNAs that are of defined length but are not transcribed from the *Y* chromosome (bands of comparable size and intensity are found in *X/Y* and *X/O* lanes): $Y_LI_{17}^-$(L) and $Y_LIII_1^+$ (L). (3) *Y* chromosomal transcripts of heterogeneous length exist beside RNAs of defined length transcribed from other chromosomes (bands of comparable size and intensity are found in *X/Y* and *X/O* lanes beside extra signals in *X/Y* lanes): $Y_LIII_1^-$ (L) and (S). The different hybridization patterns for (L) and (S) clones in this case result from partial RNA degradation in the (L) probe.

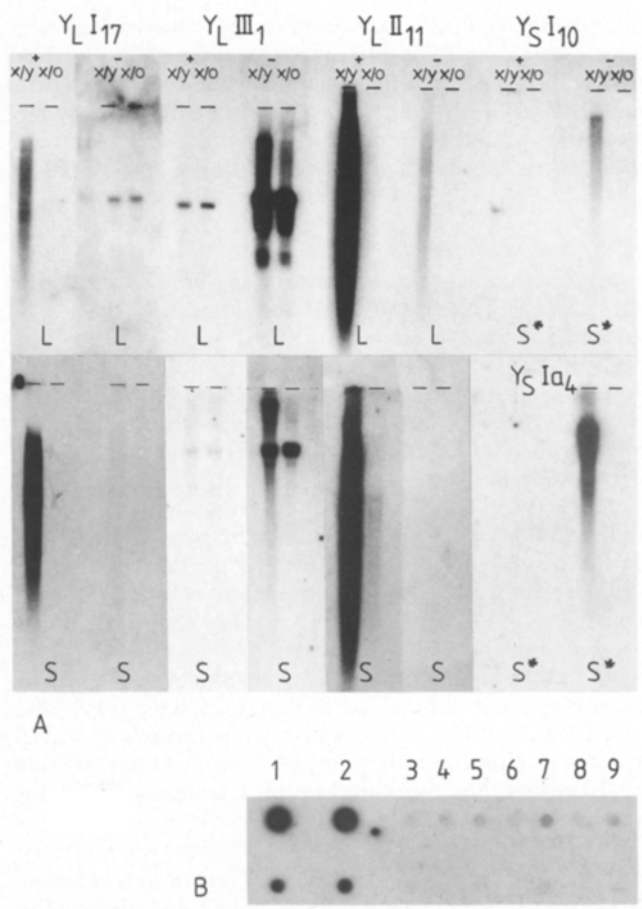


Fig. 3A, B. Analysis of transcripts from larval testes anlagen with homology to repetitive sequences on the *Y* chromosome of *Drosophila hydei*. **A** Filters with 2 µg of total RNA per lane from larval testes anlagen of *X/Y* and *X/O* genotype were hybridized in separate but otherwise identical experiments with asymmetric (+) and (−) in vitro transcripts from several *Y* chromosomal repetitive sequences. The (*S*) and (*L*) filters for a particular sequence family (Y_LI_{17}, Y_LIII_1 and Y_LII_{11}) were obtained by hybridization with the homologous short and long clones (Fig. 2), respectively. Whereas the sequences of all short clones are known (Wlaschek et al. 1988) the long clones are merely characterized by their cross-hybridization with the short ones. For Y_SI_{10} and Y_SIa_4, representing the two related subfamilies on the short arm of the *Y*, three different short clones instead of a single long one were used in separate hybridization experiments. Since all short subclones of a particular phage gave the same results only one example is depicted (*S**). **B** A filter with dots of 2 µg (upper row) and 0.2 µg (lower row) of sonicated and denatured genomic DNA of *D. hydei* females (1), males (2), and several Y_SI (3, 4, 6, 8) and Y_SIa phages (5, 7, 9) was hybridized with ^{32}P-labelled cDNA from total RNA of larval testes anlagen. The cDNA synthesis was primed by hexanucleotide random primer

In summary, the length heterogeneity observed for spermatocyte specific transcripts of all four repetitive families supports earlier results on transcription of other repetitive sequences on the *Y* chromosome (Vogt et al. 1982; Lifschytz et al. 1983; Lifschytz and Hareven 1985). Furthermore, strand specificity of transcription as first suggested by Lifschytz and Hareven (1985) supports the hypothesis of a head to tail arrangement of all repetitive elements with-

in a particular family specific sequence cluster on the *Y* chromosome. At first sight this model seems to be jeopardized by the simultaneous occurrence of similarly sized transcripts of opposite polarity from one and the same repetitive sequence, e.g. $Y_LII_{11}^+$ (L) and $Y_LII_{11}^-$ (L). This inconsistency, however, can be explained by the results of in situ hybridization experiments of ^{3}H-labelled in vitro transcripts on spermatocyte nuclei (see Fig. 4A, B).

In situ hybridization with strand-specific transcripts of Y chromosomal repetitive sequences

The in situ hybridization experiments with asymmetric (+) and (−) in vitro transcripts of our four sequence families were started primarily to detect in vivo transcripts of these sequences on the various lampbrush loops in spermatocyte nuclei. The relationship of our Y_SI and Y_SIa subclones (Wlaschek et al. 1988) to the large family of the Ns-associated clones YDh20/23 (Lifschytz et al. 1983; Lifschytz and Hareven 1985) and by9/ay1 (Vogt et al. 1982; Vogt and Hennig 1983, 1986a, b) had already been established by earlier crosshybridization experiments (kindly performed by Vogt et al.; personal communication). Therefore Y_SI_{10} and Y_SIa_4 subclones (Fig. 2) were expected to hybridize to Ns-specific transcripts (Fig. 1B, C). On the other hand, none of our other sequence families (Y_LI–YIII) showed substantial crosshybridization (kindly tested by Lifschytz et al.; personal communication) or sequence homology with the published clones YDh18 and YDh22 thought to specify the transcribed sequences on the lampbrush loops Cl and Tr, respectively (Lifschytz et al. 1983; Hareven et al. 1986). Similarly, the clones of other sequences associated with the lampbrush loops Ps and Th on the long arm of the *Y* chromosome (Hennig et al. 1983; Huijser and Hennig 1987) were not related to our isolates. Thus at the outset of this study we were faced with the problem of having to correlate three different sequence families (Y_LI–Y_LIII) with four different lampbrush loops, each already involved with transcription of at least one other repetitive *Y* chromosomal sequence family.

Unfortunately, the morphology of spermatocyte preparations is affected adversely by the fixation and hybridization procedure. For these reasons wild-type spermatocytes are not suitable for the simultaneous visualization and identification of all different *Y* chromosomal lampbrush loops. A more reliable identification of particular lampbrush loops (or of parts of them) can be obtained when the in situ hybridization experiments are performed in parallel with spermatocytes of two strains containing the complementary halves of the *Y* chromosome (combination stocks I and II, Hess 1967). One half harbours the Ns, Cl and Tr whereas the complementary half contains the Ps and Th as shown schematically in Figure 1C where the approximate location of the breakage point between both halves is indicated by the flash symbol.

All experiments with (S) and (L) clones were performed under RNA/RNA hybridization conditions according to Cox et al. (1984) as detailed under Materials and methods. The results obtained from hybridization experiments with the long clones are summarized in the two homologous panels of Figure 4 for the NsClTr and the PsTh half of the *Y* chromosome, respectively. To facilitate an understanding of the complex hybridization pattern in both panels the in situ hybridization results with a particular (+) and (−) transcript pair (indicated by + and − on top of panels) is shown adjacent to the RNA blot pattern to which this pair gives rise (reproduced from the in vitro transcripts of the (L) clones in Fig. 3A). In this way the size and distribution of each particular transcript in spermatocyte nuclei with sequence homology to our clones can be seen directly when the (+) and (−) lanes of the RNA filters in the middle are compared with hybridization patterns in the corresponding frames on the left and right side of the panels, respectively. Although the autoradiographs shown in Figure 4 are exclusively the results of in situ hybridization experiments for (L) and S*) clones, the in situ hybridizations in Figure 4A and B include the results of the corresponding (S) clones. Whether (L) and (S) probes give identical patterns of hybridization is indicated by (L) and (S) symbols within each particular frame of both panels. In those cases where (S) is missing no localized hybridization signals are detectable for (S) clones. In this way the results of both series of probes can be discussed together.

Y_SI- and Y_SIa-related sequences together represent up to 9% of *Y* chromosomal DNA (Wlaschek et al. 1988). Both subfamilies are organized in separate sequence clusters most probably situated side by side on the short arm of the *Y* chromosome (Wlaschek et al. 1988). Y_SI, the major subfamily, comprising about 75% of the total DNA in both subfamilies, contains degenerated ABB repeats of about 600 bp which are homologous to clone YDh20 (Lifschytz et al. 1983; Lifschytz and Hareven 1985). By contrast, the minor subfamily Y_SIa, equivalent to about 25% of the DNA in both subfamilies is characterized by shorter degenerated AB repeats of 400 bp homologous to clone YDh23 (Lifschytz et al. 1983; Lifschytz and Hareven 1985) and clone ay1 (Vogt and Hennig 1986a, b). In spite of their relative abundance at the DNA level only a small fraction of these sequences can be involved in transcription because the lampbrush loops Ns on the short arm of the *Y* chromosome probably do not contain more than 0.5% of *Y* chromosomal DNA (Grond et al. 1983). However, when total RNA of spermatocyte nuclei is investigated for the presence of transcripts of both subfamilies a much stronger hybridization signal is obtained for all those experiments where in vitro (−) transcripts from short Y_SIa_4 subclones (S*) were used instead of those from short Y_SI_{10} fragments (S*) (Fig. 3A). If the ^{3}H-labelled analogues of both subfamilies are used for analogous in situ hybridization experiments these differences in transcriptional activities are confirmed by the observation that hybridization to Ns transcripts is clearly restricted to $Y_SIa_4^-$ probes (S* in Fig. 4A, B: a′, b′). Indeed, the corresponding $Y_SI_{10}^-$ in vitro transcripts, although exposed for a longer time, yield unspecific background labelling for both probes (Fig. 4A, B: a, b). Logically, transcription must be restricted mainly to sequences of the Y_SIa subfamily. The weak positive reaction of Y_SI sequences on RNA filters (Fig. 3A) may be the result of substantial sequence homology between both subfamilies (Wlaschek et al. 1988). A supplementary experiment in which equal amounts of sonicated and denatured DNAs of various Y_SI and Y_SIa phages were immobilized as dots on nitrocellulose filters (dot blot) and hybridized with oligonucleotide-primed cDNA from total RNA of larval testes anlagen strongly supports the hypothesis of selective transcription of YsIa sequences. All dots of Y_SIa-related phages

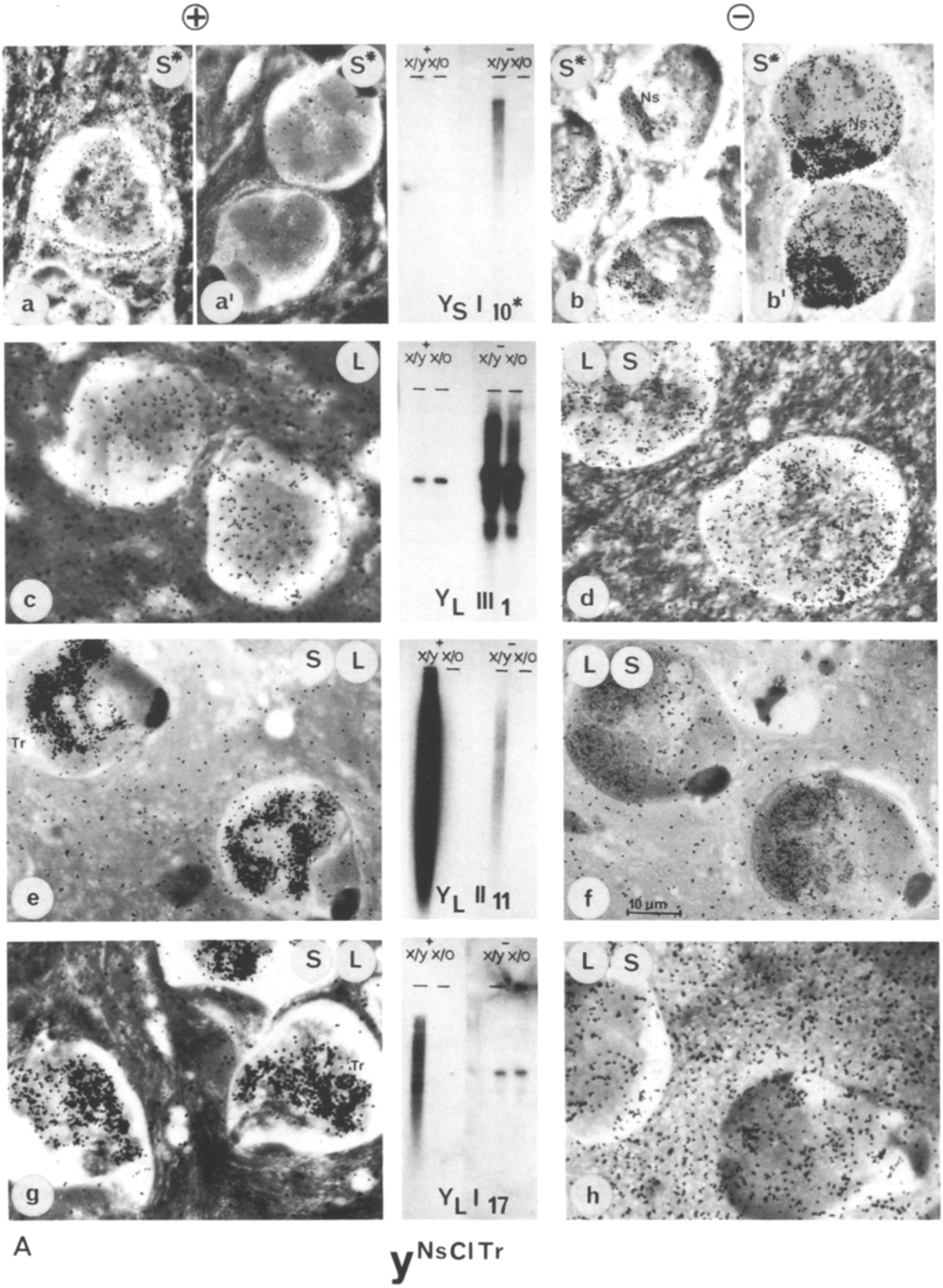
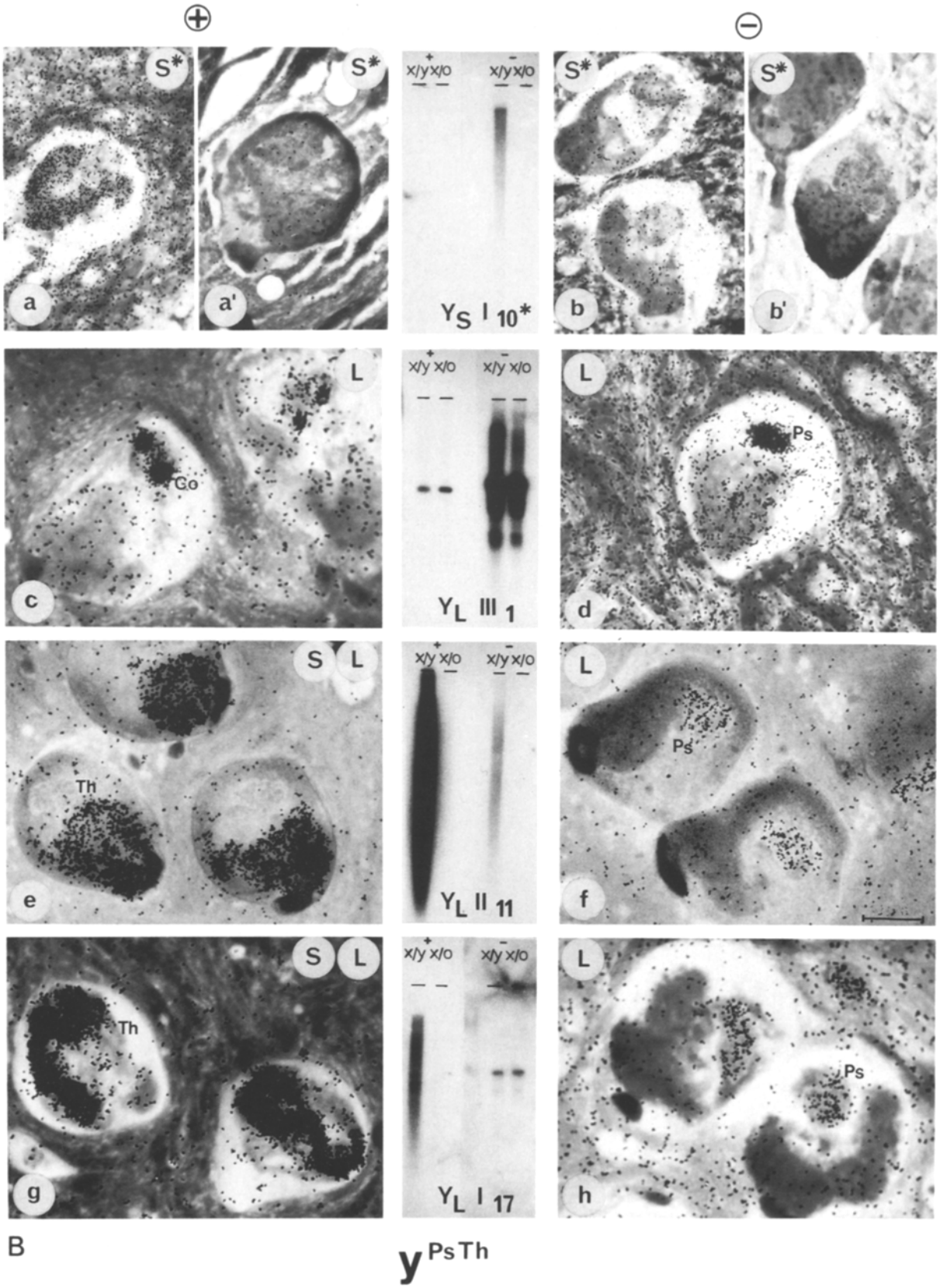


Fig. 4A, B. Localization of transcripts in nuclei of primary spermatocytes of *Drosophila hydei* by in situ hybridization with asymmetric (+) and (−) in vitro transcripts from several *Y* chromosomal repetitive sequences. **A** and **B** show the results of homologous experiments with identical sets of ^{3}H-labelled in vitro transcripts but with spermatocytes containing the Ns,Cl,Tr or the Ps,Th fragment of the *Y* chromosome, respectively (indicated by Y^{NsClTr} and Y^{PsTh} at the bottom of the panels). On the *left* and *right* side of both panels are depicted the results of experiments with (+) and (−) in vitro transcripts, respectively (indicated on the *top* of each side). The RNA filters in the *middle* were taken from Figure 3 to allow a comparison with the amount and size of the various in vivo transcripts



within the nuclei of the two different genotypes. The autoradiographs in both panels are those obtained from in situ hybridizations with in vitro transcripts of long (*L*) clones. Whether short (*S*) and (*L*) probes gave the same result with the various genotypes of spermatocytes is indicate by the *S* and *L* labels within the upper corners of each particular frame of both panels. In those cases where *S* is missing no localized hybridization signals were detectable for (*S*) clones. Although homologous experiments for small (S*) subclones from Y_SI_{10} and Y_SIa_4 were performed depicted under *a, b* and *a', b'* in both panels, respectively, the filter in the middle is that of Y_SI_{10} and therefore marked by an *asterisk*. Bar represents 10 μm

(5, 7, 9) show stronger signals than their Y_SI relatives (3, 4, 6, 8) (Fig. 3B).

The Y_LIII sequence family probably amounts to about 3% of *Y* chromosomal DNA (estimation from dot blots, Bünemann) and is located in the middle part of the long arm of the *Y* chromosome (Fig. 1C). Again *Y* chromosomal strand-specific transcripts of heterogeneous length exist. They can be identified in the *X/Y* lanes of RNA filters hybridized with (−) transcripts of $Y_L III_1$ (L) and (S) subclones and appear above a dominant RNA species of defined length visible in *X/Y* and *X/O* lanes (Fig. 3A). Whether the homologous *Y* chromosomal transcripts originate on the Ps (Fig. 4B: d) is uncertain because no such Ps-specific hybridization can be detected with the (S) clone. The RNAs of defined sizes which crossreact with (−) transcripts of $Y_L III_1$ (L) and (S) subclones are found in similar amounts in *X/Y* and *X/O* lanes (Fig. 3A). Therefore they cannot originate from *Y* chromosomal sequences. In corroboration of this finding these RNAs are distributed throughout spermatocyte cells without any visible preference for the nucleus (Fig. 4A, B: c, d). The (+) transcripts of $Y_L III_1$ (L) and (S) clones also detect a single RNA of defined size common to *X/Y* and *X/O* genotypes. The signal, however, is much weaker for the (S) probe (Fig. 3). We therefore have to assume that the twin dot like hybridization pattern (Fig. 4B: c) which is restricted to the (+) transcript of the (L) clone is caused by a sequence element within the (L) clone not present in the sequenced (S) probe. On the other hand the twin dot pattern demonstrates the accumulation of a defined RNA species in the Co, a remarkable substructure of the Ps (Fig. 1A, B).

Other unexpected results are obtained from comparable experiments with SP6 in vitro transcripts of the sequence family Y_LII. These sequences probably constitute up to 7% of *Y* chromosomal DNA (estimation from dot blots, Bünemann) and the vast majority of them are located on the distal part of the long arm (Fig. 1C). All crossreacting transcripts are of heterogeneous size and originate exclusively from the *Y* chromosome. Most of them are homologous to the (+) transcripts of $Y_L II_{11}$ (L) and (S) subclones (Fig. 3A) and are found in comparable quantities in both Tr and Th (Fig. 4A, B: e). Interestingly, lampbrush loop transcripts homologous to the (−) in vitro transcript of the $Y_L II_{11}$ (L) clone are also detected. In this case the transcripts of heterogeneous length are localized on the Ps (Fig. 4A, B: f). Since the corresponding (S) clone does not show the additional signal this discrepancy may again be assumed to result from additional sequences present in the long clone.

Characteristic hybridization patterns also arise when either of the asymmetric SP6 in vitro transcripts of Y_LI sequences are used. These sequences probably make up 3.6% of *Y* chromosomal DNA (estimation from dot blots, Bünemann) and are arranged on the long arm of the Y distally to Y_LII (Fig. 1C). A tenfold smaller amount of this sequence family is situated on the heterochromatic arm of the *X* chromosome (Wlaschek et al. 1988). The *Y* chromosomal strand-specific transcripts of heterogeneous length are detected by the (+) transcripts of $Y_L I_{17}$ (L) and (S) subclones (Fig. 3A) on Tr and Tr (Fig. 4A, B: g). But hybridization to the threads is much stronger and shows a clear lampbrush-like pattern. Otherwise, as in the case of the preceding examples, only the long (L) clone of Y_LI (−) reacts with a RNA species of defined length, not transcribed from the *Y* chromosome. This sequence is distributed throughout the whole spermatocyte cell (Fig. 4A: h) or accumulated on the Ps (Fig. 4B: h).

Discussion

The rationale for our in situ experiments is based on the assumption that each pair of family specific subclones, (L) and (S), used for the preparation of asymmetric in vitro transcripts was constructed exclusively from family specific repeats. Whereas this assumption has been verified by sequence analysis for all small (S) subclones the analogous conclusion for the longer (L) subclones, originally derived from crosshybridization experiments (Wlaschek et al. 1988), is not supported by the results of the more detailed RNA analysis presented above. In contrast, several differences in hybridization patterns for corresponding (L) and (S) probes obtained under otherwise identical reaction conditions indicate the presence of additional sequences unrelated to the family specific repeats. However, the comparison between experiments using an individual sequenced (S) clone and its longer but unsequenced (L) analogue allow us to discriminate whether the hybridization signals documented in Figures 3A and 4A, B are really caused by RNA/RNA duplex formation with in vitro transcripts of the sequenced *Y* chromosomal repeats or whether they indicate the presence of unrelated transcribed sequences within the (L) clones. Other than this, our conclusions are based on the observation that growing spermatocytes of third instar larvae and of adult testes show exactly the same nuclear structures. Therefore the assumption is made that the total RNA from larval testes anlagen, used for RNA blot analysis in Figure 3 is not substantially different from that in spermatocytes from adult testes employed for the in situ hybridizations in Figure 4A, B. Because of the substantially different in situ hybridization patterns observed for all pairs of complementary (+) and (−) in vitro transcripts in Figure 4A, B we can exclude the formation of misleading RNA/DNA hybrids. Protein/RNA complexes are also not stable under the in situ hybridization conditions used, as verified by separate filter binding assays (not shown). For these reasons the hybridization pattern of each particular in vitro transcript should be a faithful map of the distribution of all complementary RNA sequences in spermatocytes. However, at present we do not know whether all complementary sequences are accessible to the labelled probes to a comparable extent. Since a quantitative comparison of the extent of transcription of the different sequence families is severely affected by this uncertainty we will not consider it further.

Repeats of the same sequence family are transcribed on various lampbrush loops

Many of our experimental results confirm published data about transcripts of other families of repetitive DNA on the *Y* chromosome of *D. hydei* (Vogt et al. 1982; Lifschytz et al. 1983; Lifschytz and Hareven 1985; Vogt and Hennig 1986a, b; Wlaschek et al. 1988). All four sequence families show the same principal mode of transcription. Their transcripts are strand specific, heterogeneous in size and associated with defined lampbrush loop structures in spermatocyte nuclei, e.g. transcripts detected by in vitro RNA probes of $Y_S I_{10}^-$ (S*) and $Y_S Ia_4^-$ (S*), $Y_L III_1^-$ (L and S), $Y_L II_{11}^+$

(L and S), $Y_LII_{11}^-$ (L) and $Y_LI_{17}^+$ (L and S) (Fig. 4A, B). The one exception to this generalization, the Y_LII sequences, which may possibly give rise to transcripts with opposite polarities can be explained by the different hybridization pattern obtained with (+) and (−) probes (Fig. 4B: e, f). Whereas the transcripts localized in the Tr and Th show the same polarity by their hybridization to $Y_LII_{11}^+$ (L and S) probes, those on the Ps react with $Y_LII_{11}^-$ (L) and are therefore transcribed from the opposite strand. However, these results are not confirmed by experiments with the corresponding small sequenced clone (Fig. 3A). Therefore other unrelated sequence elements within the (L) clone may be responsible for the Ps-specific reaction. If the sequenced Y_LII-specific repetitive element is indeed the cause of both types of signals the above generalization about strand-specific transcription should be refined: if transcripts of opposite polarity exist for a particular repetitive *Y* chromosomal DNA they are not found within the same visible domain of *D. hydei* lampbrush loops.

A major revision must be made to the model that correlates a single family of repetitive sequences with a particular lampbrush loop in spermatocyte nuclei (Lifschytz et al. 1983). Y_LII and Y_LI sequences are clearly not restricted to a single lampbrush loop but are transcribed to a similar extent on Tr and Th (e.g. Fig. 4A, B). Since spermatocytes were used which did not simultaneously contain both *Y* chromosomal parts migration or translocation of *Y* chromosomal transcripts between the Tr and Th loops can be excluded. We cannot say at present whether Y_LI- and Y_LII-specific repeats are found within common transcripts on Tr and Th or whether each of them is restricted to smaller subdomains within one particular lampbrush loop. The isolation of a clone (dhMiF2) with weak crosshybridization to Y_LI and Y_LII phages (Huijser, unpublished observation) is in favour of mixed transcripts. Otherwise, loop transcripts with sequence homology to $Y_LI_{17}^+$ in vitro transcripts are clearly associated with the axis of the so-called proximal compact section of the Th (Thpc in Fig. 1A, B) in contrast to those homologous to the $Y_LII_{11}^+$ probe which are detected on the proximal diffuse part ot the same lampbrush loop (Thpd in Fig. 1A, B). In the case of Tr three different repetitive sequences are transcribed on one particular lampbrush loop: the 'Tr-specific' clone YDh22 (Lifschytz et al. 1983; Hareven et al. 1986) and our Y_LI and Y_LII repeats (Wlaschek et al. 1988). Recently, the number of repetitive *Y* chromosomal sequences known to be transcribed on particular lampbrush loops of *D. hydei* has been further increased by the results of Huijser and Hennig (1987). They have shown by transcript hybridization that transcripts of the so-called 'rally' sequences (a repetitive element derived from 26S rRNA) are also detected on two different lampbrush loops, Ps and Th.

Until now, no such diversity of repetitive sequences transcripts has been found among Ns-specific transcripts (Vogt et al. 1982; Lifschytz and Hareven 1985) although the clones YDh20 and YDh23 (Lifschytz et al. 1983) were originally thought to be homologous to transcripts of two different subdomains of the Ns proposed by Hess (1967). Recently, the comparison with other sequenced Ns-specific clones has shown that YDh20 and YDh23 do not represent different unrelated sequences but belong to the related subfamilies Y_SI and Y_SIa, respectively (Wlaschek et al. 1988). When the results of quantitative estimations and DNA sequence analysis of both subfamilies (Wlaschek et al. 1988) are compared with the corresponding data for their transcripts presented within the upper sections the following conclusions can be drawn. (1) The Y_SI and Y_SIa subfamilies together constitute about 9% of *Y* chromosomal DNA of *D. hydei* and are located exclusively on the short arm of the *Y*. (2) In genomic DNA Y_SI and Y_SIa sequences are found in a quantitative ratio of about 3:1. (3) Both subfamilies form separate head to tail clusters of degenerated major repeat units. (4) Transcripts originating on the lampbrush loops Ns are predominantly of the Y_SIa subfamily. (5) The transcripts are heterogeneous in length and strand specific.

When the information about transcription of all published families of repetitive DNA on the *Y* chromosome of *D. hydei* is taken and compared with the localization of transcripts along the chromosome (e.g., Fig. 1C) it becomes obvious that a particular sequence can often be found in transcripts of loops far away from the mapped position of the corresponding genomic cluster on the *Y*. Y_LI sequences, for example, are transcribed efficiently on Tr in the middle of the *Y* although the Y_LI cluster is clearly located near to the end of the long arm (Wlaschek et al. 1988). Consequently the copy number of a repetitive sequence at a certain position on the *Y* chromosome does not seem to be correlated directly with its degree of transcription on one particular lampbrush loop. More likely the opposite may be true: *only those repetitive elements which are surrounded by or intermingled with nonrepetitive sequences are found within transcripts of Y chromosomal lampbrush loops, whereas pure stretches of tandemly arranged repetitive elements form the visible clusters of transcriptionally inactive heterochromatin along the Y.*

Although this interpretation has to be proved directly by in situ hybridization on metaphase chromosomes all available data about transcription of repetitive DNA families on the *Y* chromosome of *D. hydei* are compatible with the above model. When prophase chromosomes of *D. hydei* are stained with Hoechst 33258 several fluorescent blocks can be observed along the *Y* (Bonaccorsi et al. 1981). An interpretation of this peculiar heterochromatic structure can be derived from an analogous, but much more detailed analysis of the *Y* chromosome of *D. melanogaster* (Gatti and Pimpinelli 1983), in which the dull regions between the fluorescent blocks have been correlated with various fertility genes. If this model applies to the *Y* chromosome of *D. hydei* then the dull fluorescent regions should represent the loci from which the different fertility gene-associated lampbrush loops are unfolded. A preliminary decision about the mapping of the various clusters of repetitive sequences on or between fluorescent blocks can be made from quantitative estimations. Y_SI+Y_SIa-, Y_LI-, Y-II- and Y_LIII-related sequences each amount to several percent of *Y* chromosomal DNA (see above and Fig. 1C). Consequently the amount of DNA in even a single family clearly exceeds the amount found within the longest lampbrush loops (for a recent review, see Hennig 1985). Further support for a correlation of fluorescent blocks with clusters of repetitive DNA results from the detailed studies on sequence organization in the short arm of the *Y* chromosome. All published sequences (Lifschytz et al. 1983; Vogt and Hennig 1983, 1986a, b) are members of two related subfamilies Y_SI and Y_SIa characterized by their ABB (600 bp) and AB (400 bp) repeats, respectively (Wlaschek et al. 1988). Both subfamilies are arranged in separate clusters.

Interestingly, only the minor family Y_SIa (25% of total Y_SI-related sequences) is transcribed on the Ns. But precisely the Y_SIa sequences seem to be intermingled with shorter pieces of unrelated sequences (also found in low copy numbers elsewhere in the genome of *D. hydei*) as shown by sequence analysis of the MY3 clone (Vogt and Hennig 1986a, b). Essentially the same conclusions can be drawn from our results of comparative transcript analysis for (S) and (L) clones containing the Y_LI- and Y_LIII-specific repeats (Fig. 3A). The identical pattern of crossreacting transcripts in *X/Y* and *X/O* lanes and its exclusive response to in vitro transcripts of the longer (L) clones demonstrates the presence of additional sequences within these clones. At present it is not known whether these DNA sequences which are interspersed in the repetitive repeats of the longer clones and which are also found as low copy sequences elsewhere in the genome can be detected as integral parts of the heterogeneous transcripts of the loops. Vogt and Hennig (1986a, b) succeeded in separating the ay1 repetitive sequence from its non-repetitive neighbour. Unfortunately they did not test the presence of this non-repetitive element within the Ns transcripts. Independently of a final proof for the mosaic-like structure of *Y* chromosomal fertility genes and their heterogeneous transcripts (Vogt and Hennig 1986a, b) the accumulation of transcripts of homogeneous length, originating from other chromosomes (see below), in the Ps (Fig. 4B: e) and in the Co (Fig. 4B: c) is a further indication of the structural complexity of *Y* chromosomal lampbrush loops.

Several RNAs not coded by the Y chromosome are associated with Y chromosomal lampbrush loops

Whereas our results regarding the localization of *Y* chromosomal transcripts in spermatocyte nuclei of *D. hydei* are in general accordance with most other data, our detection of the loop-specific accumulation of transcripts from other chromosomes is a novel observation. Since we do not know the complete nucleotide sequences of the longer clones used for the preparation of in vitro transcripts of the (L) type at present we cannot provide further details about the character of these sequences. However, the specific association of crossreacting RNAs with the Co and Ps is obviously (Fig. 4B: c, d, f, h) very similar to another recently published hybridization pattern (Huijser et al. 1987).

In these transcript hybridization experiments ^{3}H-labelled poly-(rC−rA) and poly-(rG−rU) polymers have been used to demonstrate strand-specific transcription of poly-(dC−dA/dG−dT) repeats on various lampbrush loops of *D. hydei*. Whereas poly(CA) produced a strong signal on the Co poly(GU) preferentially bound to the Ps. The same hybridization pattern was observed with our (+) and (−) in vitro transcripts of the Y_LIII$_1$ (L) clone (Fig. 4B: c, d). Although the authors did not investigate the size of the crossreacting RNA species the peculiar organization of Y_LIII sequences could be the reason for this phenomenon. Within the degenerated $(GTCT)_n$ sequence of the Y_LIII$_1$ clone longer stretches of $(GT)_n$ ($n=4-5$) are observed (Wlaschek et al. 1988). When the sequence polarity of both in vitro transcripts is considered then the (+) transcript contains $(CA)_n$ and the (−) strand the $(GU)_n$ stretches. If longer $(GT)_n$ stretches occur in Y_LIII it would be possible to explain why this probe and the simple heteropolymer could recognize the same RNA species. In vitro transcripts of the sequenced Y_LIII$_1$ (S) clone do not yield Ps- and Co-specific hybridization. Whether this difference between these Y_LIII$_1$ clones is caused by substantially longer stretches of $(GT)_n$ within the (L) clone cannot be decided without further sequence analysis.

At present we do not understand the significance and molecular function of the various RNA and protein species in the different lampbrush loops. In general, the protein coding capacity of all the families of *Y* chromosomal repetitive DNA is low. Moreover, these sequences have many features in common with the vast number of so-called simple sequence and satellite DNAs with unknown functions (Miklos 1985). Thus the presence of *Y* chromosomal repetitive sequences within all RNAs of heterogeneous length associated with the loops, although indicative of the *Y* chromosomal origin of these transcripts, does not prove any essential function for these repetitive elements. Indeed, several examples of satellite sequences transcribed on different lampbrush loops of the newt have been published (Varley et al. 1980; Diaz et al. 1981). In these cases simple sequence repeats are transcribed in the course of unprecisely terminated transcription of adjacent normal genes. In spite of these similarities between *Y* chromosomal repetitive DNA and analogous non-functional DNA from other sources the repetitive elements found within transcripts of *Y* chromosomal lampbrush loops may fulfil some 'gene-like' functions, perhaps by the formation of specific RNP complexes.

In support of this suggestion, the association of proteins with particular loops has been demonstrated by immunostaining with a set of specific antibodies (Hulsebos et al. 1984; Glätzer 1984; Glätzer and Kloetzel 1985, 1986). In several cases the stained loop regions coincide exactly with those labelled by in situ transcript hybridization reported here (Glätzer and Bünemann 1987). For this reason the specific morphology of the loops and their substructures has been interpreted as the visible result of the accumulation of specific RNP complexes. This specific accumulation might reflect complex formation between each of the various repetitive elements frequently found within the extended transcripts on particular lampbrush loops and a specific nuclear protein (and RNA?). At present we do not know whether the RNAs of defined length, not coded by the *Y* chromosome and homologous to in vitro transcripts of Y_LIII$^+$, Y_LIII$^-$ and Y_LI$^-$ (Figs. 3, 4) are bound to certain lampbrush loops via RNA/RNA binding due to sequence homology with *Y* chromosomal transcripts or via proteins associated with these transcripts.

Whatever the mechanism, only the detailed molecular analysis of further components involved in the complex RNP structures associated with the lampbrush loops will help to decipher the function of the 'fertility genes'. At present the work of all groups suffers from the lack of cDNA clones derived from lampbrush loop transcripts. Hence we cannot be sure that any one of our sequenced clones isolated from libraries of genomic DNAs is extensively represented in the loop-specific transcripts. A further step towards an understanding of the fertility genes would be to answer the questions of whether the loop-associated RNAs and proteins are really stored for use later in spermiogenesis (Glätzer 1984) or whether the extended loop structures play an essential role in the formation of the correct nuclear matrix within spermatocytes (Hareven et al. 1986). In addition, the processes which enable the coordinated activation and expression of these huge genes, several

thousand kilobases in length (Hackstein et al. 1982), must be studied. This activation procedure is of special interest because all fertility genes are embedded within the heterochromatin of the *Y* chromosome. Recently, evidence was presented for a gene on chromosome *3* which controls the unfolding of the complete set of *Y* chromosomal lampbrush loops (Hackstein et al. 1987).

Independently of the solution to these questions about the *Y* chromosome of *D. hydei* the spermatocyte nucleus of this fly offers a unique object for research into nuclear structures and RNP complexes in general. Its enormous size (30×50 µm) enhances the resolution of many cytological staining and labelling techniques by about one order of magnitude compared with normal cell nuclei. Most of the models of possible functions for the fertility genes on the *Y* chromosome of *D. hydei* are based on observations made possible by this special 'magnifying glass' for nuclear structures.

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