The *Y* Chromosomal Fertility Factor *Threads* in *Drosophila hydei* Harbors a Functional Gene Encoding an Axonemal Dynein β Heavy Chain Protein

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ABSTRACT

To understand the contradiction between megabase-sized lampbrush loops and putative protein encoding genes both associated with the loci of *Y* chromosomal fertility genes of Drosophila on the molecular level, we used PCR-mediated cloning to identify and isolate the cDNA sequence of the *Y* chromosomal *Drosophila hydei* gene *DhDhc7(Y).* Alignment of the sequences of the putative protein DhDhc7(Y) and the outer arm dynein β heavy chain protein DYH2 of *Tripneustes gratilla* shows homology over the entire length of the protein chains. Therefore the proteins can be assumed to fulfill orthologous functions within the sperm tail axonemes of both species. Functional dynein β heavy chain molecules, however, are necessary for the assembly and attachment of outer dynein arms within the sperm tail axoneme. Localization of *DhDhc7(Y)* to the fertility factor *Threads*, comprising at least 5.1 Mb of transcriptionally active repetitive DNA, results from an infertile *Threads*$^-$ mutant where large clusters of *Threads* specifically transcribed satellites and parts of *DhDhc7(Y)* encoding sequences are missing simultaneously. Consequently, the complete lack of the outer dynein arms in *Threads*$^-$ males most probably causes sperm immotility and hence infertility of the fly. Moreover, preliminary sequence analysis and several other features support the hypothesis that *DhDhc7(Y)* on the lampbrush loops *Threads* in *D. hydei* and *Dhc-Yh3* on the lampbrush loops *kl-5* in *Drosophila melanogaster* on the heterochromatic *Y* chromosome of both species might indeed code for orthologous dynein β heavy chain proteins.

SINCE the observation that fertility of *Drosophila melanogaster* males depends on the presence of a complete heterochromatic *Y* chromosome (Bridges 1916), intensive genetic and cytogenetic studies on *D. melanogaster* and *Drosophila hydei* revealed a comparable small number of *Y* chromosomal fertility genes in both species (Brosseau 1960; Hess and Meyer 1968; Kennison 1981, 1983). Later, cytogenetic analysis of Hoechst 33258-stained metaphases of fertile and sterile *Y* chromosomal rearrangements showed that most *Y* chromosomal fertility genes are megabase-sized (Hess and Meyer 1968; Williamson 1972; Gatti *et al.* 1976; Bonaccorsi *et al.* 1981; Hackstein *et al.* 1982; Gatti and Pimpinelli 1983; Bonaccorsi *et al.* 1988). The observation of megabase-sized transcripts associated with *Y* chromosomal lampbrush loops in primary spermatocytes further supported the idea of vastly extended transcription units for fertility genes (Glätzer and Meyer 1981; Grond *et al.* 1983). In addition, obvious similarities with respect to number and overall distribution of the fertility genes along the similar sized *Y* chromosomes in both species indicated that identically located *Y* chromosomal fertility genes might have conserved or identical functions (Gatti and Pimpinelli 1992; Hackstein and Hochstenbach 1995).

The first experimental evidence that the lampbrush loops *kl-5* and *Threads* in the subterminal regions of the *Y* chromosome in *D. melanogaster* and *D. hydei*, respectively, might harbor a gene for a dynein heavy chain protein, a vital structural component for the formation of functional outer dynein arm complexes in the sperm flagellum, came from the observation that deletions of several megabases of loop forming *Y* chromosomal DNA cause the complete loss of axonemal outer dynein arms, eliminating sperm mobility and hence fertility (Goldstein *et al.* 1982; Kociok and Glätzer, cited in Hackstein *et al.* 1991). In contrast, the observation of massive transcription of various specific satellite DNAs on all visible *Y* chromosomal lampbrush loops seemed to be incompatible with any conventional protein coding function of the corresponding fertility genes (Trapitz *et al.* 1988, 1992; Bonaccorsi *et al.* 1990; Bonaccorsi and Lohe 1991).

A feasible approach to solve this obvious incompatibility on the molecular level came forth only recently, when PCR techniques enabled amplification and partial cloning of the *Dhc-Yh3* gene present in all *kl-5*-associated *Y* chromosomal fragments of *D. melanogaster* (Gepner and Hays 1993). This finding suggested that the fertility gene encoded by *kl-5* indeed could be a gene coding for a dynein β heavy chain protein (Goldstein *et al.* 1982).

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The direct proof, however, for the presumed association of *Dhc-Yh3*-specific exons with *kl-5* loops-specific sequences required a detailed physical map of the complete *kl-5* region as well as cloned sequences from the 5′- and 3′- end of the *Dhc-Yh3* gene. Unfortunately, none of these preconditions for unravelling the molecular riddle of *kl-5* was fulfilled at the time of publication of first results on cloned P1-loop sequences of the *Dhc-Yh3* gene (Gepner and Hays 1993). Moreover, DNA satellites transcribed on the *kl-5* loops are also found in various other regions of the *Y* (Bonaccorsi *et al.* 1990; Bonaccorsi and Lohe 1991).

In contrast, physical map construction for the putative orthologous lampbrush loops *Threads* in *D. hydei* is much more advanced. In this case, a physical map spanning about 5.1 Mb of transcriptionally active *Y* chromosomal DNA in the *Threads* region has been established using pulsed field gel electrophoresis (PFGE) analysis in combination with double labeling *in situ* transcript hybridization on *Threads*-specific satellite families Y_LII, Y_LI and *rally* (Trapitz *et al.* 1992; Kurek *et al.* 1996). Based on this map, the loop morphology of wild type and several mutant *Threads* can be explained by the assumption of a single *Threads*-specific transcription unit comprising most of the *Y* chromosomal repetitive DNA between the *Pseudonucleolus Ps* and the *Nucleolus Organizer* NO_L (Figure 1). Transcription of this unit, indicated as an arrow in Figure 1A, is directed from the *Pseudonucleolus* toward the terminally located *Nucleolus Organizer* on the long arm of the *Y*. Transcripts most likely start in front of or within the 3.2-Mb region of Y_LII-related sequences, pass through subsequent blocks of 1.2 and 0.3 Mb of Y_LI- and *rally*-related sequences, respectively, and cease within or behind a smaller block of Y_LI-related repeats (Kurek *et al.* 1996). According to this map, in male sterile *Threads*$^-$ mutants loss of megabase-sized clusters of repetitive DNA and loss of the outer dynein arm in the sperm tail axoneme seem to be coupled (Figure 1, A–D). Therefore, isolation of specific sequences of the putative *Y* chromosomal dynein β heavy chain gene and the test for their presence in the *Threads*$^-$ mutant could probably answer the question whether absence of outer dynein arms in the sperm tail axoneme is indeed caused by disruption or deletion of the corresponding transcription unit on the *Y* chromosome.

To benefit from this advantage we decided to look for the putative dynein β heavy chain protein gene on the *Y* chromosome of *D. hydei*. For isolation and identification of a gene-specific probe we followed essentially the pilot experiments used for PCR-mediated cloning of partial sequences of several *D. melanogaster* genes coding for a variety of axonemal dynein heavy chain proteins (Rasmusson *et al.* 1994; Gepner and Hays 1993). Among our PCR clones we finally identified one of *Y* chromosomal origin. In this article we use detailed sequence analysis of genomic and cDNA clones to study the organization and functionality of the corresponding *Y* chromosomal gene *DhDhc7(Y)*. We present *DhDhc7(Y)* as the first example of a functional protein encoding gene on the heterochromatic *Y* chromosome of Drosophila. Alignment to GenBank sequences revealed that the putative gene product DhDhc7(Y) is most similar to the dynein β heavy chain protein DHY2 of *Tripneustes gratilla* and therefore most probably the orthologous protein in the outer dynein arm complex of the sperm tail axoneme of the fly. Since large clusters of *Threads* specifically-transcribed satellites and parts of the *DhDhc7(Y)* gene are simultaneously missing in the *Threads*$^-$ mutant, *DhDhc7(Y)*-specific sequences and *Threads*-specific satellites seem to be interspersed. Therefore it is reasonable to assume that the carboxy-terminal deletion in the dynein β heavy chain protein encoded by the *DhDhc7(Y)* gene in the *Threads*$^-$ mutant results in loss of outer dynein arms in the sperm tail axoneme and hence in sterility of *Threads*$^-$ males.

MATERIALS AND METHODS

***Drosophila hydei* stocks:** *D. hydei* stocks from our institute's collection were kept at 23° on a medium containing cornmeal, malt, sugar-beet syrup, soy flour, agar and yeast. DNA samples with Y fragments $Y^{Ns\text{-}ClTr}$ and Y^{PsTh} were obtained from females of the combination stocks KOM 697/16: attached $X/Y^{Ns\text{-}ClTr}$; $A/A \times X{\cdot}Y^{PsTh}/Y^{Ns\text{-}ClTr}$; A/A, and KOM 290/2: attached X/Y^{PsTh}; $A/A \times Y^{Ns\text{-}ClTr}\ X/A{\cdot}Y^{PsTh}$; A/A respectively (Hess 1965, 1967; Hess and Meyer 1968). X/O- and $X{\cdot}Y^{Th}/O$-males with the *Threads*-specific Y fragment Y^{Th} were prepared by crossing attached X^{wm1}/O females (Beck 1976) with wild-type males and males of stock D3F: attached $X/Y \times X{\cdot}Y^{Th}/Y$, respectively. $X/Y^{Th\text{-}}$ males missing major parts of the *Threads* were obtained from crosses of wild-type females with males of stock 99: attached $X/Y^{Th\text{-}} \times X{\cdot}Y^{PsTh}/Y^{Th\text{-}}$.

Electron microscopy: Testes were dissected and fixed in 2.5% glutaraldehyde with or without 4% tannic acid (Mizuhira and Futaesaku 1972) in 0.1 m cacodylate buffer adjusted to pH 7.4 for 2 hr at room temperature. After extensive washing with buffer solution postfixation was accomplished in 1% osmium tetroxide/cacodylate buffer for 1 hr. Tissues were dehydrated in a graded series of ethanol, transferred in propylene oxide and stepwise infiltrated with Spurr resin (SERVA, Heidelberg, Germany). Testes remained in fresh embedding medium overnight followed by polymerization at 60°. Ultrathin sections were cut with glass knives on a Reichert Ultratome OmU2 (Jung, Vienna, Austria) and stained either with lead citrate (Reynolds 1963) for 20 min or with 1% aqueous uranyl acetate for 30 min. Electron micrographs were taken on an EM 300 electron microscope (Philips Technologies, Cheshire, CT).

Genomic DNA preparation and Southern blot analysis: Genomic DNA was prepared from adult flies essentially as described in Scott *et al.* (1983) and Rasmusson *et al.* (1994). DNA samples (18 μg per lane) were digested with restriction enzymes, separated in 0.5% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) according to standard methods (Sambrook *et al.* 1989). Membranes were UV-crosslinked with 80 mJ in a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Hybridizations with ^{32}P-labeled *DhDhc7(Y)* and autosomal *DhDhc3* [at 33A according to the polytene chromosome map of Berendes (1963)] probes were performed overnight in 2× SSC, 0.2% SDS, 1× Denhardt's, and 50 μg/ml sonicated salmon sperm

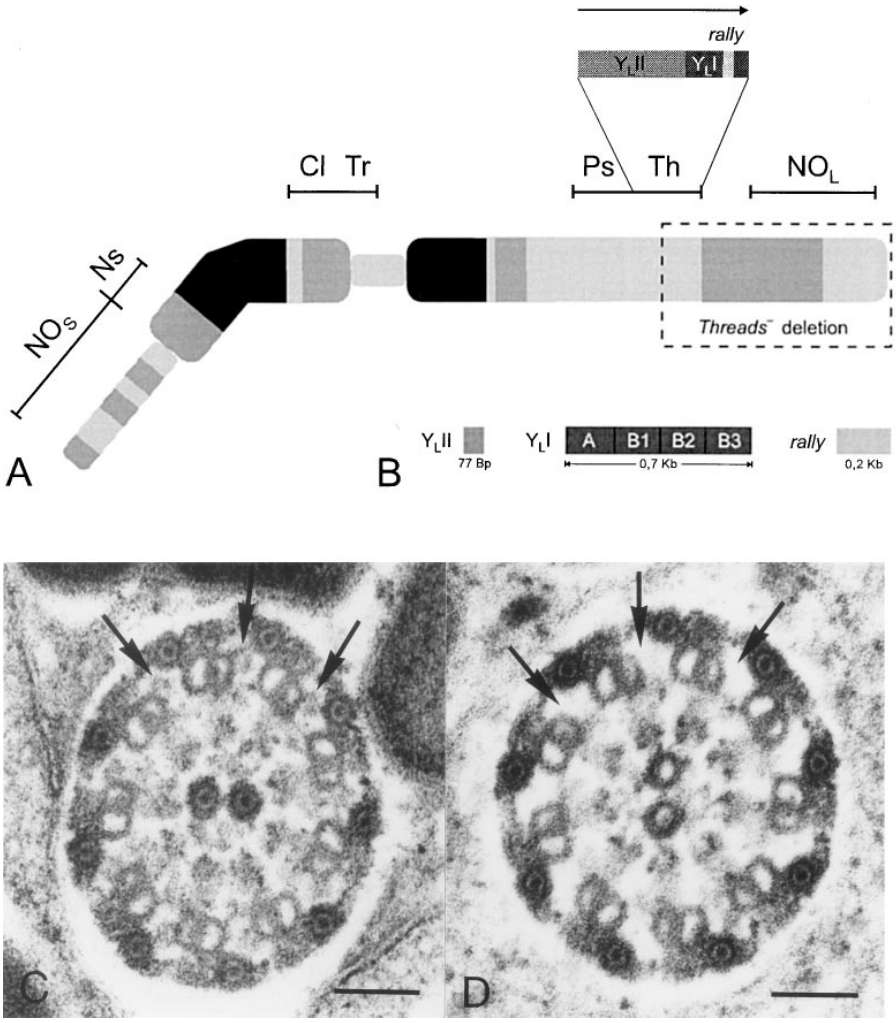

Figure 1.—Overview of cytogenetic and molecular data regarding the correlation of satellite DNA transcription on the *Threads* lampbrush loops on the *Y* chromosome of *D. hydei* with the presence of outer dynein arms within the spermtail axoneme. (A) Schematic drawing of the *D. hydei Y* chromosome after staining with Hoechst 33258 (Bonaccorsi *et al.* 1981). Light-grey areas represent nonfluorescent, grey areas weakly fluorescent and black areas brightly fluorescent regions. Cytogenetic location of five fertility gene-associated lampbrush loops visible in primary spermatocytes, *Nooses* (*Ns*), *Clubs* (*Cl*), *Tubular ribbons* (*Tr*), *Pseudonucleolus* (*Ps*) and *Threads* (*Th*), is taken from Hackstein (1987). NO_L and NO_S show the positions of the *Nucleolar Organizers* on the long and short arm of the *Y*. The arrow indicates the *Threads*-specific transcription unit comprising at least 5.1 Mb of repetitive DNA organized in four different clusters as derived by Kurek *et al.* (1996). The *Y* chromosomal part deleted in infertile *Threads*$^-$ males is marked by a box of broken lines. (B) Schematic illustration of singular family-specific basic repeats of *Threads*-specific repeat families Y_LII, Y_LI and *rally* (Huijser and Hennig 1987; Wlaschek *et al.* 1988). (C and D) Cross-sections of sperm tails of a *D. hydei* wild type and a *Threads*$^-$ male, respectively. The arrows point to hook-like outer dynein arms attached to the A tubule of the wild-type axoneme microtubule doublets (C). The corresponding areas of the *Threads*$^-$ genotype appear empty (D). Bar, 50 nm.

DNA. Membranes were washed at high stringency with 0.1× SSC, 0.1% SDS at 65° for 2 × 10 min. Films were exposed at −80° in the presence of two enhancer screens (DuPont, Wilmington, DE).

Library construction of mechanically sheared genomic *D. hydei* male DNA: DNA from *D. hydei* males was sheared by 250 passages through a syringe needle (25 gauche 1) and size fractionated on a preparative agarose gel. Fragments of 5.5–10 kb were eluted from the gel and treated with Mung Bean Nuclease and Klenow fragment for creation of blunt ends. The blunt ended fragments were then ligated to *Eco*RI/*Xho*I-Adaptors (Stratagene) and cloned into the *Eco*RI site of Lambda ZAP II *Eco*RI CIAP (Stratagene). Transformation yielded 1.15×10^6 primary plaques.

PCR amplification of DNA sequences for Drosophila dynein heavy chain proteins: In summary, PCR amplifications for cloning of DNA sequences encoding the conserved P1-loop region of dynein heavy chain proteins were performed with combinations of three different sets of sense and antisense primers (Figure 2A) on genomic DNA samples of *D. hydei* and *D. melanogaster* males: sense primers: Dyn-5′, (5′ACCCCCCT GACCGACCGCTGCTAC3′); Dyn-ITP, (5′AT(A/C/T)ACNC CNCT(C/G/T)ACNGA(C/T)(A/C)G3′); Dyn-GPA, (5′GGA CCAGCCGGAACTGGAAAAACGG′); antisense primers: Dyn-3′, (5′GTTGAACTCGTCGAAGCAGCCCCA3′); Dyn-FIT, (5′ CCNGG(A/G)TTCATNGT(A/G/T)AT(A/G)AA3′); Dyn-HYD, (5′GCICGIAGICCCCA(A/G)TC(A/G)TA(A/G)TG3′). The different primer combinations were applied under reaction con-

ditions as described previously by Rasmusson *et al.* (1994). PCR-derived clones of different experiments were picked at random and sequenced. Finally clones for eight different dynein heavy chain genes were obtained from both Drosophila species (Figure 2B).

Cloning and sequencing of *DhDhc7(Y)*: A survey of the various cloning steps is shown in Figure 4. The main body of *DhDhc7(Y)*-specific cDNA sequences was derived from the genomic clones DhDhc7-g1, DhDhc7-g6 (Figure 4A) and Dh7G5/2, Dh7G5/3 and Dh7G5/4 (Figure 4B), which were obtained by screening a Lambda GEM-11 library prepared from a *Mbo*I partial digest of *D. hydei* DNA and a Lambda ZAPII library obtained from mechanically sheared DNA (see above), respectively, with *DhDhc7(Y)*-specific subclones according to standard procedures (Sambrook *et al.* 1989). Regions in the 5′-part of the *DhDhc7(Y)* cDNA not covered by the genomic clones were derived from a set of five overlapping RACE clones, Dh7RACE 1–5 (Figure 4D), generated by five subsequent RACE reactions (see below). To assign intron/exon junctions correctly in cases where cDNA sequences were derived from genomic sequences, open reading frames in sequenced subclones were aligned with the deduced amino acid sequences of corresponding regions of the axonemal dynein β heavy chain DYH2 from sea urchin embryo (Gibbons *et al.* 1991). All putative splice junctions within the *DhDhc7(Y)* gene were proven by RT-PCR amplification (Kawasaki 1988) with specific primer pairs on samples of testes mRNA of *D. hydei*. The last small sequence gap at the 3′-end of the *DhDhc7(Y)* cDNA comprising the putative amino acids 4532–4564 was closed by clone DhDhc7-c1 (Figure 4E), which resulted from PCR on DNA samples from a cDNA library constructed of *D. hydei* testes poly(A)$^+$-RNA in Lambda ZAPII (*ZAP-cDNA* Synthesis Kit, Stratagene). PCR amplification (Figure 6B) was performed using the universal primer (5′GTA AAACGACGGCCAGT3′) specific for pBluescript-phagemid specific sequences in Lambda ZAPII phage vectors in combination with the sense primer DhDhc7-E29-5′ (5′GCTGTAACA CAGGATAAACAGG3′) complementary to sequences in the last but one exon of *DhDhc7(Y)*. Finally, sequences of the 3′-terminal exon of *DhDhc7(Y)* of clone DhDhc7-c1 were used for designing of specific primers for two separate supported PCR experiments (see below). The resulting clones, Dh7G3/1 and Dh7G3/2 (Figure 4E), were needed to extend the genomic sequences from the 3′-end region of *DhDhc7(Y)* further into the antisense (intron I^{4532}) and sense direction, respectively (Figure 7B).

RACE: The 5′-end of *DhDhc7(Y)* cDNA comprising 87 bp of the 5′-UTR and of the coding region for amino acids 1–1264 was cloned (in five overlapping clones Dh7RACE 1–5; Figure 4D) by five subsequent RACE reactions using the 5′/3′ RACE-Kit (Boehringer Mannheim, Mannheim, Germany) in combination with sets of three nested primers: Dh7RACE 1: Dh7RACE-2 (5′CATCAATAAGCTCATAGCAATTAGGGCAA GGAACAG3′); Dh7RACE-2A (5′CACTTGATAGCTTTGTA TGGGTGC3′); Dh7RACE-1A (5′CTCAGTAT-CCCTAAACTC GTATCC3′); Dh7RACE 2: Dh7RACE-3 (5′GCAAAGAAGAC AAAGCCTGCTTTGGAATTGGGGTC3′); Dh7RACE-3A (5′AC AACGATTGGTTCCTGAAGCTCC3′); Dh7RACE-3B (5′CTT CCAACCGTAAGTAAGTAACGC3′); Dh7RACE 3: Dh7RACE-4 (5′GCACCATTCTGCAATTCTTTCCAATTTAATTCGAGC3′); Dh7RACE-4A (5′AAGATTCGGTGTTAGTTCACATTCC3′); Dh7 RACE-4B (5′CAGAAAGATTGGCCTCCTCATCTG3′); Dh7 RACE 4: Dh7RACE-5 (5′GGCTGAAATTTGTCGACCTTTAA GACCACCTACGAC3′); Dh7RACE-5A (5′TCCAATTTCATAAA ATCACGGCCAG3′); Dh7RACE-5B (5′GCTTGCCATGTCCAA TCTGTGAG3′); Dh7RACE 5: Dh7RACE-6 (5′CTATACGAG GTGCTTCCTTCATGACTTCATCAATGC3′); Dh7RACE-6A (5′ CTGAGCTATACCATTACGCATCTC3′); Dh7RACE-6B (5′AT GTCGTTCACAATTACTCTAGTC3′).

Cloning of genomic sequences by supported PCR: Supported PCR (sPCR) enables efficient amplification of genomic DNA sequences flanking short stretches of a known sequence (Rudenko *et al.* 1993). Here sPCR was applied to enable the extension of the small 3′-terminal exon into both directions. Extension into 5′- and 3′-direction was performed in two separate reactions with primer DhDhc7-3-3′: (5′CTGTAAGAG TAAACAAACTCC3′) and DhDhc7-3-5′B: (5′GAGCAAAGAA CGGGATAGCAAGTG3′), respectively (Figure 6B). In a first step 10 μg of sonicated *D. hydei* male DNA was biotinylated in 50 μl reaction mixture under the following conditions: 4 μl 25 mm $MgCl_2$, 1 μl 20 μg/μl DhDhc7-3-3′ or (DhDhc7-3-5′B) specific primer, 2 μl dNTP-mixture (1 mm dATP, dCTP, dGTP each and 0.65 mm dTTP + 0.35 mm bio16-dUTP) and 0.3 μl *Taq* DNA polymerase (1.5 units) in the buffer recommended by the supplier. Polymerization was achieved after denaturation of template DNA for 2 min at 92°, followed by primer annealing for 2 min at 50° and extension for 10 min at 72° with subsequent cooling to 25°. After ethanol precipitation the DNA pellet was resuspended in 50 μl 10 mm Tris/HCl, 1 mm EDTA, 100 mm NaCl, pH 7.5, mixed with 5 μl streptavidin magnetic particles (Boehringer Mannheim) and incubated for 30 min at room temperature. Particles with bound biotinylated DNA were separated by a particle separator, washed two times with 10 mm Tris/HCl, 1 mm EDTA, 1 m NaCl, pH 7.5, and resuspended in 20 μl H_2O. Ligation with double-stranded linker primer LP1-*Taq*-primer (5′ACCGTG GATCAGTACCGCGACTTGT3′) and LP1-short-primer (5′C AAGTCGCGGT3′) was performed in a total volume of 25 μl by addition of 0.5 μl (0.5 μg) LP1-*Taq*-primer, 0.25 μl (0.25 μg) LP1-short primer, 2.5 μl ligase buffer (10×) and 2 μl (10 units) T4 DNA ligase (Boehringer Mannheim) and incubation for at least 14 hr at 12°. For final PCR amplification, particles were washed two times with *Taq* polymerase buffer and resuspended in 18 μl H_2O. Particle aliquots of 6 μl were mixed each with 5 μl 10 × *Taq* polymerase buffer, 1 μl dNTP mixture (10 mm), 3 μl 25 mm $MgCl_2$, 1 μl LP1-*Taq*-primer (20 ng/μl), 1 μl DhDhc7-3-3′ or (DhDhc7-3-5′B) primer (20 ng/μl) and 0.3 μl (1.5 units) *Taq* DNA polymerase (Promega, Madison, WI). After initial template denaturation at 92° for 5 min PCR amplifications were performed using the following program: 35 cycles of 2 min at 50°, 3 min at 72°, 1 min at 93°, and a final 10 min extension at 72°. Amplified fragments were cloned in the pGEM-T-vector (Promega) and several independent 5′- and 3′-specific extension clones were sequenced. The largest clones Dh7G3/1 and Dh7G3/2 contained inserts of 289 and 271 bp, respectively (Figure 4E).

Detection of *DhDhc7(Y)* and *DhDhc3*-specific sequences in samples of genomic DNA: The following four pairs of sense and antisense oligonucleotides were used for gene-specific detection of *DhDhc7(Y)* sequences by PCR amplification on genomic *D. hydei* DNA. Positions of primers relative to the coding region within the cDNA of *DhDhc7(Y)* are given in parentheses: sense: Dh7gDNA-145-5′ (-143) - 5′ACTAAACG GGAAAGTAGGC3′- (-126), antisense: Dh7AA40-3′ (123) - 5′GGTTGTTATACACTTTGTCC3′- (104); sense: Dyn-ITP (5758) - 5′AT(A/C/T)ACNCCNCT(C/G/T)ACNGA(C/T) (A/C)G3′- (5777), antisense: DhDhc7-3′ (6118) - 5′TCG CTCGTAGGGCAATGTAT3′- (6098); sense: DhDhc7-3-5′ (13,595) - 5′ATTACGTGGGCCCACTTATG3′- (13,614), antisense: DhDhc7-3-3′ (13,689) -5′CTGTAAGAGTAAACAAACT CC3′- (13,669); sense (intron I^{4532}): DhDhc7-3I-5′C -5′CT TGAGTGCTCTCATTTCTTTC3′- (Figure 7B), antisense (nontranscribed): DhDhc7-3-3′B -5′GAGTACTTAGTTATG TATAAGG3′ (Figure 7B). Since major parts of the *DhDhc3* cDNA used in control reactions are presently unknown, nucleotide positions of *DhDhc3*-specific primers are taken from the corresponding *DhDhc7(Y)* sequences by aligning the sequences

B

D. hydei	*D. melanogaster*
DhDhc0 : Dyn-ITP / Dyn-HYD	**Dhc36C : Dyn-ITP / Dyn-FIT**
DhDhc1 : Dyn-ITP / Dyn-FIT	**Dhc62B : Dyn-ITP / Dyn-FIT**
DhDhc2 : Dyn-GPA / Dyn-HYD	DmDhc1 : Dyn-GPA / Dyn-HYD
DhDhc3 : Dyn-ITP / Dyn-HYD	**Dhc93AB: Dyn-ITP** / Dyn-HYD
DhDhc4 : Dyn-ITP / Dyn-HYD	**Dhc98D : Dyn-GPA** / Dyn-HYD
DhDhc5 : Dyn-ITP / Dyn-FIT	?
DhDhc6 : Dyn-ITP / Dyn-FIT	**Dhc64C : Dyn-ITP / Dyn-FIT**
DhDhc7(Y): Dyn-ITP / Dyn-HYD	**DhcYh3 : Dyn-ITP / Dyn-FIT**
?	**Dhc16F : Dyn-ITP / Dyn-FIT**

Figure 2.—Illustration of dynein heavy chain P1-loop-specific primer pairs and cloned sequences obtained by PCR amplification of genomic DNA of *D. hydei* and *D. melanogaster*. (A) Location of PCR primers (indicated by arrows) relative to the ATP-binding site (P1-loop) of sea urchin dynein β heavy chain protein DYH2 (shaded area). The depicted amino acid sequence comprises residues 1827 to 2028 of the β heavy chain protein DYH2 of axonemal outer dynein arm from the sea urchin (Gibbons *et al.* 1991). For primer sequences, see materials and methods. (B) Pairwise sorting of PCR amplification products for putative orthologous dynein heavy chain protein genes in *D. hydei* and *D. melanogaster*. Bold letters indicate *D. melanogaster* clones already described (Rasmusson *et al.* 1994). Primer pairs used for PCR-mediated cloning of each particular gene are indicated. The actual size of particular PCR clones can be derived from the positions of the corresponding primer pairs in the sea urchin protein sequence in A.

of their putative protein products: sense: Dh3E04A-5′ (1566) -5′CGATTGCCACAATCTAGAGAG3′- (1586); antisense: Dh3 E07A-3′ (2037) -5′GTTGAGGATCAACGAGTGTAC3′- (2018).

RESULTS

PCR screenings for *Y* chromosomal dynein heavy chain genes yielded sequences for at least seven pairs of orthologous genes in *D. hydei* and *D. melanogaster*: Initially, sequences of our oligonucleotide primer pair Dyn-5′ and Dyn-3′ for PCR amplification of the putative *Y* chromosomal *D. hydei* dynein β heavy chain gene were based exclusively on conserved amino acid sequences of nucleotide binding (P1-loop) regions in two different dynein heavy chain genes: axonemal dynein β heavy chain of *T. gratilla* (Gibbons *et al.* 1991) and cytoplasmic dynein heavy chain of *Dictyostelium discoideum* (Koonce

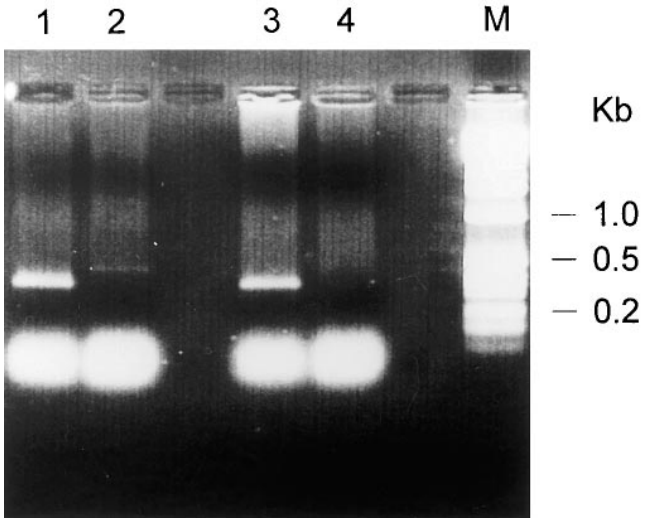

Figure 3.—Documentation of *DhDhc7(Y)*-specific sequences in *Y* chromosomal fragments in different genotypes of *D. hydei* by PCR with Dyn-ITP and the gene-specific primer DhDhc7-3′ for the P1-loop region of dynein β heavy chain (Figure 2A). PCR was performed on DNA samples from: lane 1, wild-type males; lane 2, wild-type females; lane 3, Y^{PsTh} males and lane 4, Y^{NsCITr} males (for chracterization of lampbrush loops, see Figure 1A). M, Molecular weight markers: 1-kb ladder (GIBCO-BRL, Gaithersburg, MD).

et al. 1992). Although oligonucleotides were designed by using the codon usage table of *D. melanogaster* as cited in Ashburner (1989), not more than a single dynein heavy chain gene, *DhDhc0*, was detected.

Primer pairs for a more efficient PCR amplification of a broader spectrum of dynein heavy chain-specific sequences became feasible, however, in 1994 on the basis of published nucleotide sequences for P1-loop regions of multiple heavy chain genes in sea urchin (Gibbons *et al.* 1994) and in *D. melanogaster* (Rasmusson *et al.* 1994). The pair of degenerated primers Dyn-ITP/Dyn-FIT (Rasmusson *et al.* 1994) seemed to be especially well suited for amplification of multiple heavy chain genes of *D. melanogaster* including the *Y* chromosomal *Dhc-Yh3* gene (Gepner and Hays 1993). To identify the putative *Y* chromosomal gene in *D. hydei* we explored the efficiency of this and other primer combinations in simultaneous PCR assays on genomic DNA samples of *D. melanogaster* and *D. hydei*.

The resulting genes and corresponding primer pairs used for PCR amplification are summarized in Figure 2. Alignment of deduced amino acid sequences and conserved sites of small introns enabled identification of putative orthologous gene pairs in both species (Figure 2B). We found for *D. melanogaster* essentially the published set of genes (Rasmusson *et al.* 1994) as indicated by bold letters in Figure 2B. For *D. hydei* a comparable number of different dynein heavy chain genes was obtained. In several cases an additional degenerated 3′-primer Dyn-HYD enabled further extension of cloned

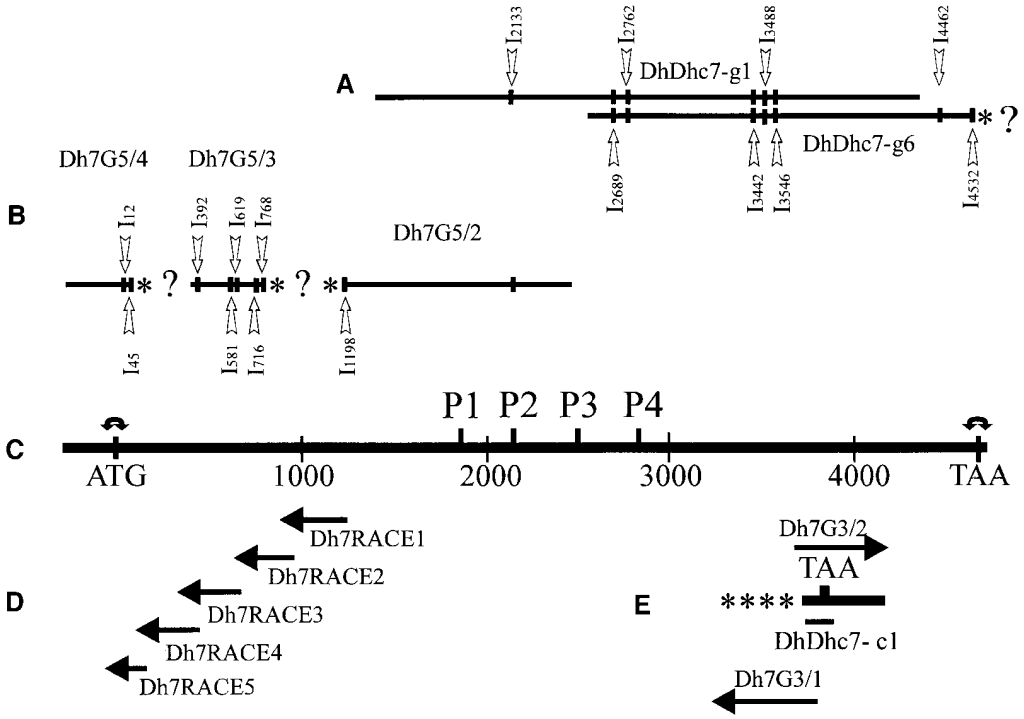

Figure 4.—Schematic overview for the assembly of *DhDhc7(Y)* cDNA from a collection of various *DhDhc7(Y)*-specific primary clones. (A) Overlapping inserts of the genomic λGEM11 phages DhDhc7-g1 and DhDhc7-g6 comprising 21 kb genomic DNA with seven complete introns (I^{2133} to I^{4462}) and an asterisk in place of 7 kb of intron I^{4532}. The question mark indicates a gap in the gene due to the unknown total size of intron I^{4532}. (B) Lambda ZAPII phages Dh7G5/2, 5/3 and 5/4 containing nonoverlapping genomic DNA fragments of mechanically sheared *D. hydei* male DNA (material and methods) with at least five complete introns (I^{12} and I^{392} to I^{716}) and three partial introns I^{45}, I^{768} and I^{1198}. The introns are specified according to their positions within the putative DhDhc7(Y) protein. Sequences of partial introns are not shown but marked by asterisks. Question marks indicate two gene gaps of unknown extension between introns I^{45} and I^{392} and I^{768} and I^{1198}. (C) The assembled cDNA of *DhDhc7(Y)* messenger is shown and characterized by amino acid numbers for the open reading frame which specifies the DhDhc7(Y) protein. Translation start and stop codons are indicated as ATG and TAA, respectively. Two-headed bent arrows above ATG and TAA symbolize the *DhDhc7(Y)* regions amplified by 5′-end 3′-end specific PCR primer pairs, respectively (Figure 8). DNA P1 to P4 indicate ATP binding sites as derived from sequence alignment (Figure 7) with the dynein β heavy chain protein DYH2 of *T. gratilla* (Gibbons *et al.* 1991). The transcription start is presently unknown. Therefore the sequences preceding the ATG might contain some additional nontranscribed DNA from the promoter region. The 3′-end also extends into the untranscribed region (see Figure 6B). (D) Size and extension of five overlapping RACE clones Dh7RACE1–5 obtained by five subsequent RT-PCR cycles. (E) 5:1 enlargement of the 3′-end of the cDNA in (C) to indicate the contribution of PCR clone DhDhc7-c1 and two supported PCR clones, Dh7G3/1 and Dh7G3/2, to the assembly and extension of sequences in the 3′-region of *DhDhc7(Y)*, as shown in more detail in Figure 6B.

sequences for about 180 bp into the 3′-region (Figure 2A). In summary, we detected one novel *D. melanogaster* gene *DmDhc1* but missed the orthologous partners for *Dhc16F* and *DhDhc5* in *D. melanogaster* and *D. hydei*, respectively. Sequence data of all non-*Y* chromosomal dynein heavy chain genes of *D. hydei* will be published in a more evolutionary context elsewhere. According to sequence criteria *DhDhc7(Y)* of *D. hydei* and *Dhc-Yh3* of *D. melanogaster* could be assumed to represent orthologous *Y* chromosomal genes.

Localization of *DhDhc7(Y)* on the *Y* chromosome of *D. hydei*: To confirm the *Y* chromosomal location of *DhDhc7(Y)* and to assign the gene to a particular subregion on the *Y* we used the available sequence information for the synthesis of a gene-specific primer DhDhc7-3′ (Figure 2A), which allowed, in combination with primer Dyn-ITP, selective PCR amplification of *DhDhc7(Y)* sequences. PCR on DNA samples of different *D. hydei* strains with various parts of the *Y* chromosome mapped *DhDhc7(Y)* to the subterminal region on the long arm of the *Y* (Figure 3). The *DhDhc7(Y)*-specific PCR amplification product of 360 bp was restricted to DNA samples comprising the distal part of the long arm of the *Y* chromosome (Figure 1A) specified by the lampbrush loops *Pseudonucleolus (Ps)* and *Threads (Th)*. In contrast, the fragment was not observed with female DNA and in DNA samples containing the short arm of the *Y* with lampbrush loops *Nooses (Ns)* and the proximal part of the long arm containing the loops *Clubs (Cl)* and *Tubular ribbons (Tr)* (Figure 1A).

Genomic clones DhDhc7-g1 and DhDhc7-g6 contain 21 kb of *DhDhc7(Y)* specific DNA: Because a testis-specific cDNA library of *D. hydei* did not exist at that time, a radiolabeled probe of clone PCR-DhDhc7(Y) was used

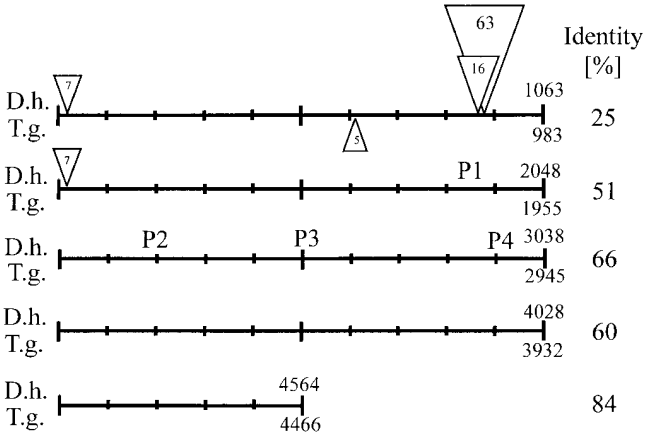

Figure 5.—Schematic alignment of the putative DhDhc7(Y) protein of *D. hydei* with the dynein β heavy chain protein DHY2 of *T. gratilla* (Gibbons *et al.* 1994). The aligned regions of *D. hydei* and *T. gratilla* proteins are specified by the numbers of amino acids above and below the end of each line, respectively. Numbers to the right represent the percentage of identical amino acids for corresponding parts of both proteins except sequences put in triangles. Numbers in triangles above and below the lines indicate insertions larger than four amino acids within the *D. hydei* and the *T. gratilla* protein, respectively. It is obvious that similarity of both proteins is lowest by far in the N-terminal region. According to the numbers in triangles the actual size difference of about hundred amino acids between both proteins is mainly the result of two insertions of 16 and 63 amino acids in the N-terminal region of the fly protein. The P-loop positions are indicated by P1–P4 (see Figure 4C). The corresponding DNA sequences are available under the accession No. AF031494 (*D. hydei*) and No. X59603 (*T. gratilla*).

to screen a genomic library of *Mbo*I partial digests of *D. hydei* male DNA in Lambda GEM11 vector. In the course of two subsequent screens we obtained two phages, DhDhc7-g1 and DhDhc7-g6, with overlapping inserts of 13.7 and 15.3 kb, respectively, covering about 21 kb of continuous *Y* chromosomal DNA (Figure 4A). Restriction fragments comprising the complete DNA inserts of both phage were subcloned into plasmid pBluescriptII KS and sequenced by standard methods (Sambrook *et al.* 1989). Map construction of the *DhDhc7(Y)* gene was enabled by alignment of translated putative open reading frames of particular subclones with corresponding amino acid sequences of the dynein β heavy chain gene *DYH2* of *T. gratilla* (Gibbons *et al.* 1994). Inserts of both phage together comprised genomic sequences coding for an open reading frame of 3167 amino acids, seven complete introns, I^{2133}–I^{4462}, ranging in size from 47 to 3130 bp and an incomplete intron I^{4532} of at least 7 kb (Figure 4A). The sequence of the putative protein derived from this reading frame is similar and colinear to a stretch of 3165 amino acids from the carboxy-terminal part of the sea urchin dynein β heavy chain protein DYH2 (Figure 5).

Further support for the functionality of the putative *DhDhc7(Y)* gene came from a series of RT-PCR experiments with specific intron-flanking primer pairs on testes RNA, which indicate perfect splicing of all putative introns originally derived solely from sequence comparison with *DYH2* of *T. gratilla*. In addition, *Y*-specificity of *DhDhc7(Y)* was verified by Southern blot analysis of male and female DNA by hybridization with a *DhDhc7(Y)*-specific subclone of 1.9 kb (Figure 6B). A 2.1-kb probe of the homologous region of the autosomal dynein heavy chain protein gene *DhDhc3* was applied to the same filter as a control (Figure 6C).

Although these results indicated the presence of an active gene encoding an axonemal dynein β heavy chain protein on the heterochromatic *Y* chromosome of Drosophila as predicted (Hardy *et al.* 1981; Goldstein *et al.* 1982; Kociok and Glätzer, cited in Hackstein *et al.* 1991; Gepner and Hays 1993), definite proof would only come from analysis of cDNA sequences. Unfortunately, we were unable to isolate any additional *DhDhc7(Y)*-specific clones from our library of *Mbo*I partially digested male DNA in Lambda GEM11, although we applied a variety of gene-specific subclones in combination with different hybridization conditions. For these reasons we were forced to use alternative approaches for completing the *DhDhc7(Y)* sequences into 5′- and 3′-direction.

Clone DhDhc7-c1 defines the 3′-end of *DhDhc7(Y)*: To close the small gap at the 3′-end comprising a putative stretch of about 32 amino acids, PCR was applied on DNA samples from our only recently established cDNA library of *D. hydei* testes poly$(A)^+$-RNA in Lambda ZAPII. PCR amplification was performed with the universal primer for pBluescript-phagemid sequences in Lambda ZAPII phages and primer DhDhc7-E29-5′ (Figure 7A) for exon-specific sequences from the 5′-flanking region of intron I^{4532}. Sequencing of the 0.3-kb insert of the resulting clone DhDhc7-c1 (Figure 7A) revealed several characteristic features for the 3′-end of a typical messenger RNA: codons for 32 additional amino acids at the carboxy-terminus were followed by a TAA stop codon, two overlapping polyadenylation signals and a short poly(A)-tail (Figure 7A). Since an exon-specific single *Apa*I site in DhDhc7-c1 (Figure 7A) is absent from the insert of phage DhDhc7-g6, the residual 7 kb of the phage insert must comprise noncoding intron I^{4532}-specific DNA of *DhDhc7(Y)* (Figure 4, A–E). This conclusion could be verified by sequence analysis (data not shown).

Sequences from the 5′-region of *DhDhc7(Y)* mRNA are not present in a cDNA library of *D. hydei* testes poly$(A)^+$-RNA in Lambda ZAPII: Although we originally constructed the cDNA library of *D. hydei* testes poly $(A)^+$-RNA especially for completion of *DhDhc7(Y)* cDNA sequences we were unable to detect any crossreacting clones in this library in spite of several screens with various subclones derived from the 5′-region of genomic clone DhDhc7-g1 (Figure 4A). Otherwise, the library was prepared by priming the cDNA reaction with oli-

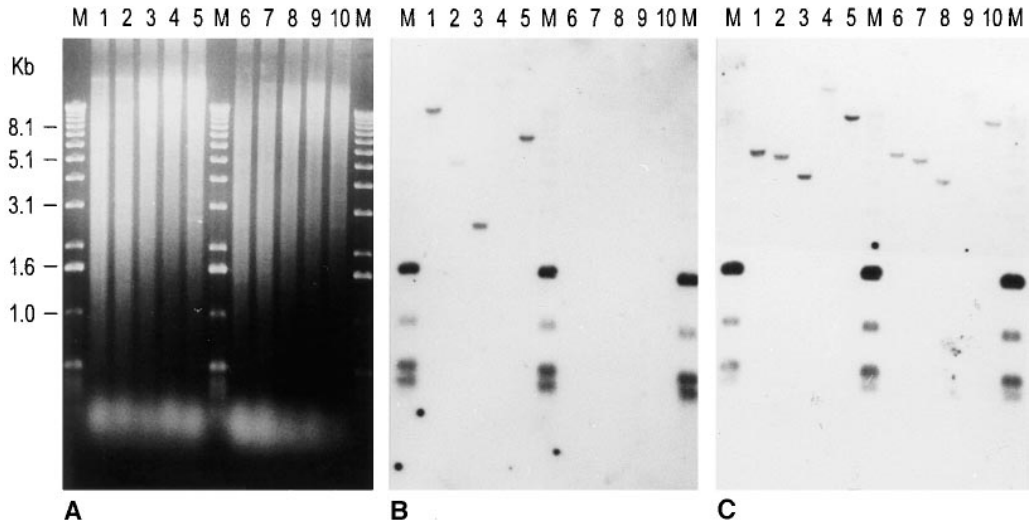

Figure 6.—Southern blot showing the *Y* chromosomal location of *DhDhc7(Y).* Equal amounts of male (lanes 1–5) and female (lanes 6–10) DNA of *D. hydei* were digested with *Hin*dIII (lanes 1 and 6), *Pst*I (lanes 2 and 7), *Sac*I (lanes 3 and 8), *Sca*I (lanes 4 and 9) and *Xba*I (lanes 5 and 10) and submitted to gel electrophoresis. The ethidium bromide stained gel (A) was blotted and subsequently hybridized with similar-sized probes from homologous regions (between intron I^{1198} and P1-loop in Figure 4) of the *Y* chromosomal gene *DhDhc7(Y)* and the autosomal gene *DhDhc3* in (B) and (C), respectively. Whereas the autosomal gene gives the same pattern of DNA fragments for males and females, *DhDhc7(Y)*-specific fragments are male-specific. M, Molecular weight marker: 1-kb ladder (GIBCO-BRL).

go(dT) and random oligonucleotide hexamers. For this reason, specific absence of clones from the 5′-end of *DhDhc7(Y)* messenger RNAs was interpreted as the result of RNase contamination during the poly(A)$^+$ preparation. Because mRNAs of dynein heavy chain proteins are about 14,000 nucleotides in length they are more susceptible to RNase contaminations than RNAs of normal size.

A library of sheared genomic *D. hydei* DNA in Lambda ZAPII phages led to detection of genomic *DhDhc7(Y)*-DNA associated with repetitive sequences: Because of the severe underrepresentation of sequences from the 5′-end of *DhDhc7(Y)* in our *D. hydei* testes cDNA library, our strategy for cloning of additional sequences from the 5′-region of *DhDhc7(Y)* was based on the observation that sequence conservation in the 5′-third of dynein β heavy chain protein genes from different species is generally too poor to allow the deduction of reliable degenerated PCR primers (Koonce *et al.* 1992; Mitchell and Brown 1994). In addition, we took into account that subclones from the 5′- and 3′-ends of the genomic phages DhDhc7-g1 and DhDhc7-g6, respectively, gave negative results in all library screens, but nevertheless produced reasonable hybridization signals with genomic DNA fragments >100 kb on PFGE Southern blots (data not shown). This behavior can be expected from hybridization with probes from exons flanking large introns composed of extended stretches of repetitive DNA resistant to digestion by most restriction enzymes. For the same reason, genomic sequences associated with longer stretches of tandemly repeated DNA are also severely underrepresented in common restriction fragment-based libraries (Burgtorf and Bünemann 1994). To overcome this problem with cloning of repetitive DNA we constructed a novel genomic library of mechanically sheared *D. hydei* male DNA (5.5–10 kb DNA fragments) in Lambda ZAPII phages (materials and methods).

Our interpretation, that the negative screening results on restriction fragment-based genomic libraries might be caused mainly by intragenic clusters of repetitive DNA, found direct support by sequence analysis of the 7-kb insert of phage Dh7G5/2 (Figure 4B), which had been picked from the Lambda ZAPII library by screening with a subclone derived from the 5′-end of *DhDhc7(Y)*-specific sequences in phage DhDhc7-g1. Whereas most of the sequences in Dh7G5/2 were overlapping with those already cloned in DhDhc7-g1, the insert also provided some new protein encoding sequences from the 5′-region of *DhDhc7(Y)* up to amino acid 1198 followed by about 500 bp of intron I^{1198} (asterisk in Figure 4B). Intron-specific sequences, however, were made of 167-bp unique sequences immediately adjacent to the exon/intron border and a stretch of 18 $CA(GT)_n$-repeats (n = 3–14) representing the 5′-end of the *DhDhc7(Y)* insert in phage Dh7G5/2.

Five overlapping RACE clones contain the missing 1197 codons from the 5′-part of *DhDhc7(Y)*: Since most of our difficulties seemed to result from the presence of repetitive sequences in *DhDhc7(Y)* clones in genomic libraries we decided to apply reverse transcriptase (RT)-PCR for cloning of additional sequences from the 5′-third of the putative *DhDhc7(Y)* cDNA. Finally, completion of sequences from the 5′-region of the

DhDhc7(Y) cDNA was enabled by five overlapping clones, Dh7RACE1–5 (Figure 4D), resulting from a series of five successive 5′-RACE reactions on *D. hydei* testes RNA. Together, the partial cDNA sequences comprised 87 nucleotides of the putative leader followed by 1264 codons specifying a single open reading frame with weak but obvious sequence similarity to the N-terminus of the dynein β heavy chain protein DYH2 of *T. gratilla* (Figure 5). To determine the organization of *DhDhc7(Y)*-specific sequences on the genomic level, the Lambda ZAPII library was screened again in two separate experiments with mixures of labeled inserts of clones Dh7RACE2/ Dh7RACE3 and Dh7RACE4/Dh7RACE5, respectively. However, only two single clones, Dh7G5/3 and Dh7G5/4, were obtained. Together, both clones comprised 5.4 kb of genomic DNA with five complete small introns (I^{12} and I^{392} to I^{716}) and two (I^{45} and I^{768}) of unknown length (Figure 4B). Because phage Dh7G5/4 also contained 1002 bp upstream to the ATG codon, their alignment with the 87 bp of the putative leader sequences in clone Dh7RACE5 showed the absence of any further introns at least for the already cloned part of the 5′-untranslated region (UTR).

Assembly of all *DhDhc7(Y)*-specific clones revealed an open reading frame for a dynein β heavy chain protein of 4564 amino acids: Although three gaps of unknown size were still left in the noncoding genomic sequences of gene *DhDhc7(Y)* (question marks in Figure 4, A and B), all available coding sequences of *DhDhc7(Y)* could be assembled into a single cDNA of 13,856 nucleotides (accession No. AF031494) with a large single open reading frame of 4564 amino acids. The full-length sequence similarity of the putative protein DhDhc7(Y) with the dynein β heavy chain protein DYH2 of *T. gratilla* (Figure 5), strongly suggests that the lampbrush loops *Threads* on the heterochromatic *Y* chromosome of *D. hydei* indeed contain a structural gene for an axonemal dynein heavy chain protein as predicted from other results on *Y* chromosomal *Threads* deletions (Kociok and Glätzer, cited in Hackstein *et al.* 1991). Additionally, physical mapping experiments demonstrated that the lampbrush loops *Threads* are primarily the product of transcription processes along a continuous stretch of *Y* chromosomal DNA comprising at least 5.1 Mb of repetitive sequences (Kurek *et al.* 1996). The apparent colocalization of two transcription processes on the *Threads* comprising extended areas of satellite DNAs as well as protein-encoding sequences raise, once more, the question for their linear arrangement.

Sequences from the 3′-end of *DhDhc7(Y)* are missing in the mutant *Threads*$^{-}$: Availability of a sterile mutant, *Threads*$^{-}$, and genomic sequences from the 5′- and 3′-ends of *DhDhc7(Y)* facilitated an experiment to directly localize the *DhDhc7(Y)*-specific transcription unit with respect to putative satellite-specific transcription unit(s) on the *Threads*. Since *Threads*$^{-}$ males are lacking the outer dynein arms in their sperm tail axonemes (Figure 1D) as well as major parts of *Threads*-specific repetitive sequences (Figure 1A and Kurek *et al.* 1996), both *Threads*-specific transcription units seemed to be affected simultaneously. To study whether the complete *DhDhc7(Y)* transcription unit is present on the *Y* of

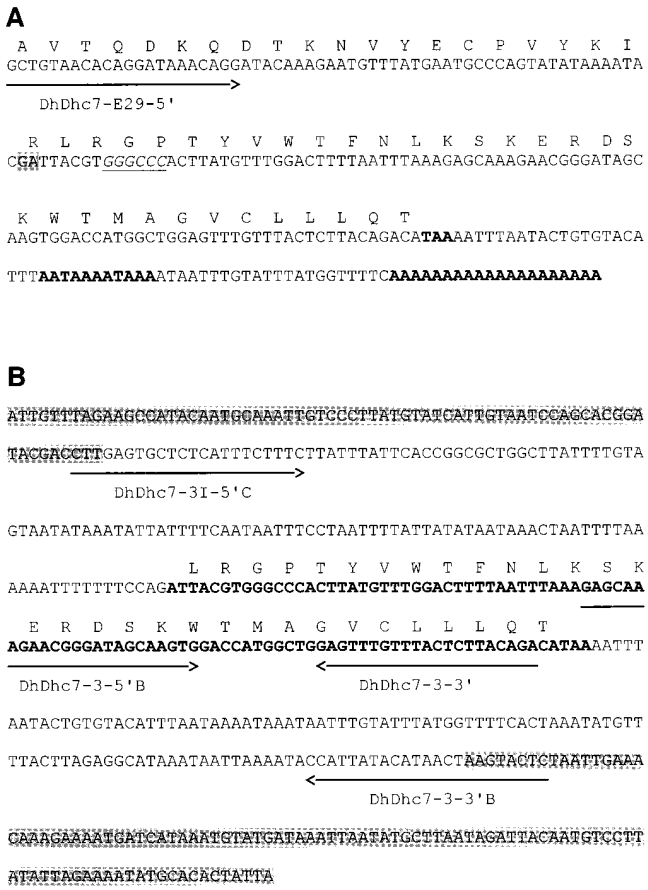

Figure 7.—Overview of cloned sequences specifying the 3′-end of the *DhDhc7(Y)* transcription unit. (A) DNA and deduced amino acid sequence of PCR-mediated clone DhDhc7-c1 comprising the 3′-terminus of *DhDhc7(Y)* mRNA (see Figure 4E). Arrow DhDhc7-E29-5′ indicates the position of the PCR 5′-primer which was applied in combination with the phagemid universal primer as 3′-primer on DNA samples from a cDNA library constructed of *D. hydei* testes poly(A)$^{+}$-RNA in Lambda ZAPII. Location of exon/exon splice junction is indicated by the shaded GA. *Apa*I site is underlined and shown in italics. The TAA stop codon, two overlapping putative polyadenylation signals and the poly(A) tail are in bold letters. (B) Schematic overview of genomic DNA sequences in the region of the 3′-end of *DhDhc7(Y)* as derived from supported PCR clones Dh7G3/1 and Dh7G3/2 (Figure 4E). The corresponding sPCR amplifications were mediated by the PCR primers DhDhc7-3-3′ in 3′- and DhDhc7-3-5′B in 5′-direction, respectively (for details, see materials and methods). Shaded sequences show regions in both clones with sequence similarity to different parts of 28S ribosomal genes. Arrows DhDhc7-3I-5′C and DhDhc7-3-3′B show the extension and position of the primer pair which was used for PCR amplification of a 345-bp fragment of the 3′-region of *DhDhc7(Y)*. This sample was used as a probe for mapping the 3′-end of *DhDhc7(Y)* transcription unit by PCR on DNA samples of flies with different parts of the *Y* chromosome (Figure 8).

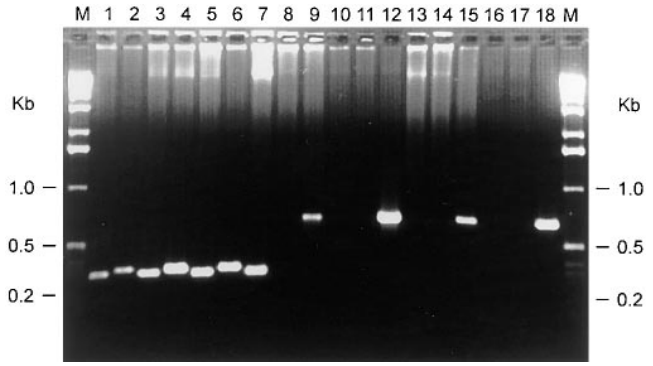

Figure 8.—PCR-mediated mapping of the *DhDhc7(Y)*-specific transcription unit on different *Y* chromosomal fragments. Lanes 1–18 contain the amplification products which were obtained on genomic DNA samples containing different parts of the *Y* chromosome. Each particular *Y* chromosomal fragment is characterized by presence or absence of the five lampbrush loops *Ns*, *Cl*, *Tr*, *Ps* and *Th* (see Figure 1A). Lanes 1 and 2 ($X/Y^{Ns\text{-}Cl,Tr,Ps,Th}$); 3 and 4 ($X\cdot Y^{Ps,Th}$); 5 and 6 ($X\cdot Y^{Th}$); 7–9 *Threads*$^{-}$ ($X\cdot Y^{Ps}$); 10–12 ($X/Y^{Ns\text{-}Cl,Tr}$); 13–15 (X/O); 16–18 (X/X). PCR amplifications in lanes 1, 3, 5, 7, 10, 13, and 16 were performed with the primer pair Dh7gDNA-145/5′/Dh 7AA40-3′ for specific amplification of a 320-bp fragment from the 5′-region of the *DhDhc7(Y)* transcription unit (symbolized by a two-headed bent arrow above ATG in Figure 4C). The corresponding primer pair Dh7-3I-5′C / Dh7-3-3′B for amplification of a specific 345-bp fragment from the 3′-end of the *DhDhc7(Y)* transcription unit (symbolized by a two-headed bent arrow above TAA in Figure 4C) was used for samples in lanes 2, 4, 6, 8, 11, 14, and 17. Controls specifying a 666-bp fragment of the autosomal *DhDhc3* gene were primed with 5′-primer Dh3E04A-5′ and 3′-primer Dh3E07A-3′ and applied to DNA samples in lanes 9, 12, 15, and 18. It is evident that the 3′-region of *DhDhc7(Y)* is absent in DNA of *Threads*$^{-}$ males in lane 8 but present in $X\cdot Y^{Th}$ males in lane 6. M: Molecular weight markers: 1-kb ladder (GIBCO-BRL).

Threads^{-} males we designed two primer pairs for specific PCR amplification of sequences from 5′- and 3′-end of the *DhDhc7* cDNA. The amplified regions are symbolized by two-headed bent arrows above the ATG and TAA codons (Figure 4C).

Whereas the amplification of the 320-bp fragment (−144 to 175) of the 5′-region specified by the primer pair (Dh7gDNA-145-5′ and Dh7AA40-3′) worked immediately, the small size of 3′-end-specific sequences (13,595 to 13,688) available from clone DhDhc7-c1 (Figures 4E and 6A) seemed to prevent the generation of any defined DNA fragment independent of the presence of *Y*-specific DNA. In order to extend the size of 3′-end-specific sequences of *DhDhc7(Y)* we applied supported PCR technology on samples of *D. hydei* male DNA (materials and methods). Antisense specific extension (into intron I^{4532}) with primer DhDhc7-3-3′ produced clone Dh7G3/1 with 195 bp of intron-specific sequences, whereas sense-specific priming by DhDhc7-3-5′B yielded clone Dh7G3/2 with 157 additional base pairs into the 3′-untranscribed region. In fact, however, only 126 and 55 bp of these intron- and 3′-region specific sequences, respectively, could be used in the course of PCR amplification of a more extended 3′-end specific DNA fragment because both clones ended in different stretches of degenerated ribosomal RNA genes (shaded regions in Figure 7B). To avoid any cross-reaction with ribosomal RNA genes the 3′-specific primer pair (DhDhc7-3I-5′C and DhDhc7-3-3′B) for amplification of a 345-bp fragment was designed to cover 129 bp of intron I^{4532}, 97 bp [32 translated codons specifying the carboxy-terminus of DhDhc7(Y)], 56 bp of the 3′-UTR and 63 bp of untranscribed DNA further downstream (Figure 7B).

As controls, samples of all DNAs were submitted to separate PCR amplifications with an additional primer pair (Dh3E04A-5′ and Dh3E07A-3′). This primer pair was designed for specific amplification of a 666-bp fragment [homologous to the nucleotides from 1566 to 2050 in *DhDhc7(Y)*] from an autosomal dynein heavy chain protein gene *DhDhc3* located on chromosome *2* (33A) of *D. hydei* (A. Reugels, unpublished results). The results of parallel PCR experiments with one *DhDhc3* and the two *DhDhc7(Y)*-specific primer pairs are clear cut: whereas lanes of the *Threads*$^{-}$ mutant DNA contain gene *DhDhc3* as well as the 5′-end of gene *DhDhc7(Y)*, they clearly lack the 3′-end of the *Y* chromosomal gene (Figure 8). Consequently, loss of the tip from the long arm of the *Y* chromosome, comprising the complete NO_L and major parts of several clusters of *Threads*-specific repetitive sequences (Figure 1A and Kurek *et al.* 1996), also includes the loss of some sequences from the 3′-end of the *DhDhc7(Y)*-specific transcription unit, which code for at least 32 amino acids from the carboxy terminus of the axonemal outer arm dynein β heavy chain protein DhDhc7(Y).

DISCUSSION

In spite of several earlier indications that the so-called fertility genes on the *Y* chromosome of Drosophila could also harbor “normal” structural genes, encoding axonemal dynein proteins (Hardy *et al.* 1981; Goldstein *et al.* 1982), this interpretation of the nature of the fertility genes had not been fully accepted in the past for the following reasons: (i) Activity of most fertility genes on the *Y* causes the unfolding of large lampbrush-like loops in primary spermatocytes; (ii) megabase-sized transcripts on these loops consist essentially of different types of tandemly-arranged more or less simple repeats (Wlaschek *et al.* 1988; Hennig *et al.* 1989; Trapitz *et al.* 1988, 1992; Bonaccorsi *et al.* 1990; Bonaccorsi and Lohe 1991; Kurek *et al.* 1996) interspersed with middle repetitive transposon-like sequences (Lankenau *et al.* 1989, 1994; Huijser *et al.* 1988), (iii) sensitivity against mutagens is hundred- to thousand-fold in comparison with normal euchromatic genes of Drosophila (Judd *et al.* 1972; Hardy *et al.* 1981; Gatti and Pimpinelli 1983; Hackstein *et al.* 1982, 1991).

With respect to these criteria, cloning of the almost complete cDNA of *DhDhc7(Y)* represents an important step towards an understanding of structure and function of *Y* chromosomal fertilitity genes of Drosophila. The mere existence on the heterochromatic *Y* chromosome of an open reading frame of 13,692 bp encoding one of the largest eukaryotic protein species known so far is intriguing and an indirect proof for the functionality of this gene. Additional support for a functional role of *DhDhc7(Y)* results from the analysis of cDNA clones derived from testes RNA. All introns (at least 16) known so far are perfectly spliced and the 3′-UTR exhibits all features of a normal messenger RNA (Figure 7A). Although the exact transcription start site is still unknown, similar-sized 5′-UTR regions of about 90 bp in several independent RT-PCR clones indicate that the complete leader might have approximately this length. In addition, alignment of the sequences of the putative protein DhDhc7(Y) and the outer arm dynein β heavy chain protein DYH2 of *T. gratilla* shows that there is similarity along the entire length of the protein chains (Figure 5). The alignment also corroborates earlier observations that sequence differences between orthologous heavy chain proteins are much more pronounced in the amino terminal third of the molecule (Koonce *et al.* 1992; Mitchell and Brown 1994). Whether two insertions of 16 and 63 amino acids in the fly protein indicate a special subtype of outer arm dynein β heavy chains in the very extended *D. hydei* sperm (23 mm!!) is an open question and awaits the analysis of orthologous proteins from other Drosophila species. In summary, however, outer arm dynein-specific β heavy chain proteins can be assumed to fullfill orthologous functions within sperm tail axonemes of both species.

This is supported by the observation that a lack of outer arm dynein complexes in sperm tail axonemes is the characteristic phenotype of *D. hydei Threads*$^-$ males (Figure 1D). *Threads*$^-$ mutants, however, are also characterized by a simultaneous loss of megabase-sized *Threads*-specific satellite clusters (Kurek *et al.* 1996; Figure 1A) and of at least 345 bp from the 3′-region of the *DhDhc7(Y)* transcription unit (Figure 8). The deletion includes the already cloned 195 bp of the 3′-end of intron I^{4532} as well as exon sequences encoding the last 32 amino acids at the carboxy-terminus of DhDhc7(Y), the complete 3′-UTR and 157 bp of untranscribed sequences further downstream (Figure 7B). Due to this breakpoint within intron I^{4532} and the concomitant deletion of all more distally located *Y* chromosomal sequences (Kurek *et al.* 1996), translation of *DhDhc7(Y)* mRNA into a functional protein is impossible in the *Threads*$^-$ mutant. Consequently, outer dynein arms cannot be formed because the assembly of this multiprotein complex is not possible in the absence of dynein β heavy chain proteins (Dutcher 1995). In summary, our analysis supports former conclusions that have been derived from biochemical and electron microscopic experiments on *Y* chromosomal deletions mainly affecting the fertility gene *kl-5* of *D. melanogaster* (Hardy *et al.* 1981; Goldstein *et al.* 1982). Males with deletions on the long arm of the *Y* chromosome affecting the region of the fertility gene *kl-5* and *Threads* are sterile because of sperm immotility caused by the lack of outer dynein arms in the axonema of their sperm tails. According to these criteria *kl-5* and *Threads* are most probably orthologous *Y* chromosomal genes.

Despite the colocalization of a functional protein encoding gene and a *Y* chromosomal fertility gene, Hennig (1993) is also right with his argument that the mere presence of a protein encoding gene would explain neither the extreme size of *Y* chromosomal genes, as derived from genetic experiments (Gatti *et al.* 1976; Bonaccorsi *et al.* 1981; Hackstein *et al.* 1982; Gatti and Pimpinelli 1983; Bonaccorsi *et al.* 1988) nor their intimate association with the formation of extended and species-specific lampbrush loops (Hess and Meyer 1968). Although *DhDhc7(Y)* codes for a rather large protein of 4564 amino acids, its size alone can account, at best, for a tenfold increase of sensitivity against mutagens in comparison to proteins of average size. Otherwise, simultaneous deletion of large clusters of *Threads*-specifically transcribed satellites and *DhDhc7(Y)*-specific sequences in the *Threads*$^-$ mutant shows that both transcription processes are intimately linked. Unfortunately, a direct *in situ* transcript hybridization experiment with a *DhDhc7(Y)* exon probe on spermatocytes of $X{\cdot}Y^{Th}$ males failed to produce any *Threads*-specific signals. This negative result, however, can be easily explained by the fact that any *DhDhc7(Y)* exon-specific sequence is part of a huge transcript of several Mb more or less evenly distributed over the whole *Threads* region (Kurek *et al.* 1996). As a consequence the corresponding signal will be too faint to be discernible from any unspecific background. Over the years several *Threads*$^-$ mutants have been isolated. All of them are missing the terminal part of the *Y* including the NO_L, the complete Y_LI and *rally* clusters, but only parts of the large Y_LII cluster (Figure 1A). For these reasons we do not believe that *Threads*$^-$ mutants involve any rearrangements of *DhDhc7(Y)* exons. In addition, signal distribution resulting from a comparable *in situ* transcript hybridization with Y_LII-specific probes on spermatocyte nuclei of *Threads*$^-$ males favors a normal transcription of the residual clusters of Y_LII satellites in spite of the loss of the complete distal end of the *Y* chromosome (Kurek *et al.* 1996).

At present, our results on the *Threads*$^-$ mutant can be interpreted by several models. On a first look, most features of the *Threads*$^-$ mutant can be explained by a single chromosomal break within intron I^{4532} of a separate *DhDhc7(Y)*-specific transcription unit inserted somewhere between at least two separate clusters of Y_LII repeats, similar to the island model of Le *et al.* (1995). In this case, transcription of repetitive and unique sequences on the *Threads* could occur independently from

each other and *DhDhc7(Y)*-specific active transcription could also provide passive transcription of more distally located satellite specific sequences, comparable to transcription of histone genes in oocytes of the newt Notophthalmus (Diaz *et al.* 1981). Unfortunately, fertile flies without unfolded *Threads* have never been observed (Hackstein *et al.* 1982). It is incorrect to assume, however, that active transcription of megabase-sized clusters of repetitive DNA might be necessary for initiation of transcription of a more distally located gene; this assumption does not explain the extreme size of lampbrush loops associated genes.

A second model, which assumes that cotranscription of satellite- and *DhDhc7(Y)*-specific sequences is the result of some dispersed intra-intronic repeats, comparable to the situation in the heterochromatic gene *light* in *D. melanogaster* (Devlin *et al.* 1990a,b) is also very unlikely. According to PFGE analysis and *in situ* transcript hybridization on several *Threads* mutants, $Y_L II$, $Y_L I$ and *rally* repeats form small numbers of large fragments of several hundred kilobases in size (Trapitz *et al.* 1988, 1992; Kurek *et al.* 1996). These clusters are most likely transcribed unidirectionally into continuous transcripts of at least 5.1 Mb (Kurek *et al.* 1996).

We prefer a third model introduced recently by Hackstein and Hochstenbach (1995), which places extended clusters of *Threads*-specifically transcribed repetitive DNA and *DhDhc7(Y)*-specific sequences into one and the same megabase-sized transcription unit resulting in 5.1 Mb primary transcripts for a mRNA of a 527-kD protein. The following arguments support this model: (i) Whereas most *DhDhc7(Y)*-specific introns are in the size of 47–65 bp and can be easily bridged by PCR-mediated amplification with specific primer pairs, similar experiments are completely unsuccessful for a small number of introns (question marks in Figure 4); (ii) sequences of these introns are rare or even essentially absent from genomic DNA libraries; (iii) in the few cases, where sequences of these introns have been detected, the corresponding clones are unstable and/or contain tandem repeats; (iv) labeled probes which are derived from exon-specific sequences from flanking regions of these introns produce hybridization signals in the size range of several hundred kilobases on PFGE Southern blots of genomic *D. hydei* DNA (A. Reugels, unpublished data); (v) a single megabase-sized *Threads*-specific transcription unit would immediately explain the extraordinary size of *Y* chromosomal fertility genes.

The last argument is the most attractive one, because it would bring the somewhat mysterious *Y* chromosomal fertility genes of Drosophila back to the rules of classic genetics. Even with unusual megabase-sized introns of repetitive DNA, they would nevertheless no longer represent "a new type of eukaryotic genes" (Hennig *et al.* 1989). The main advantage of this model of a single *Threads* lampbrush loops specific transcription unit is that it is provable. This can be done by PFGE analysis on Southern blots of genomic DNA digested with specially selected restriction enzymes. In the case of large introns of repetitive DNA all enzymes, which do not cut the tandem repeat and possess only a low number of known sites within the *DhDhc7(Y)* transcription unit, should produce DNA fragments of several hundred kb. In this case only a single fragment should cross-react with the *DhDhc7(Y)*- as well as with the satellite-specific probe.

Whether an even more convincing hybridization experiment with two differentially labeled DNA probes specific for both ends of *DhDhc7(Y)* on metaphase chromosomes will produce two separate signals depends mainly on the actual intron position. It will not work, *e.g.*, in the case of a megabase-sized intron at the place of I^{4532} in *D. hydei*, because a hybridization probe of sufficient size for the 3′-end of *DhDhc7(Y)* is not available due to the direct neighborhood of degenerated rRNA repeats (Figure 7B). A similar argument regarding the size of suitable probes might also be true for the two putative large introns in the 5′-region of *DhDhc7(Y)* indicated as question marks in Figure 4B. Therefore, any conclusive hybridization experiments with *DhDhc7(Y)*-specific probes on metaphase chromosomes of *D. hydei* must be based on prior positive results of corresponding PFGE experiments. But, if *kl-5* and *DhDhc7(Y)* are indeed orthologous genes in *D. melanogaster* and *D. hydei*, respectively, with similarly constructed lampbrush loops, the hybridization experiment could also be done on metaphase chromosomes of *D. melanogaster*. *D. melanogaster* would provide the additional advantage of a large number of available *Y* chromosomal translocations with known breakpoints also in the *kl-5* loops region (Gatti and Pimpinelli 1983, 1992). Presently, both experimental approaches are being undertaken simultaneously and will most likely result in a definite (and common?) solution for the longstanding riddle of lampbrush loops-associated fertility genes on the *Y* chromosomes of different Drosophila species.

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