Drosophila melanogaster kl-3 and kl-5 Y-loops harbor triple-stranded nucleic acids

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Summary

Primary spermatocyte nuclei of *Drosophila melanogaster* contain three prominent lampbrush-like loops. The development of these structures has been associated with the transcription of three fertility factors located on the Y chromosome, named kl-5, kl-3 and ks-1. These loci have huge physical dimensions and contain extremely long introns. In addition, kl-3 and kl-5 were shown to encode two putative dynein subunits required for the correct assembly of the sperm axoneme. Here, we show that both the kl-5 and kl-3 loops are intensely decorated by monoclonal antibodies recognizing triple-stranded nucleic acids, and that each loop presents a peculiar molecular organization of triplex structures. Moreover, immunostaining of *Drosophila hydei* primary spermatocytes revealed that also in this species – which diverged from *D. melanogaster* 58 million years ago – Y-loops are decorated by anti-triplex antibodies, strongly suggesting a conserved role of loop-associated triplexes. Finally, we showed that in *D. melanogaster* wild-type lines that are raised at the non-permissive temperature of 31±0.5°C (which is known to induce male sterility in flies) both the triplex immunostaining and the axonemal dynein heavy chains encoded by kl-3 and kl-5 are no longer detectable, which suggests a functional correlation between loop-associated triplexes, the presence of axonemal proteins and male fertility in fly.

Key words: Lampbrush-like loops, Triplex, Heterochromatin, Y chromosome, *Drosophila hydei*.

Introduction

The Y chromosome of *Drosophila melanogaster* is a entirely heterochromatic chromosome comprising ~12% of the male genome (Pimpinelli et al., 1976). The main genetic function of this chromosome is male fertility. Bridges demonstrated that X0 flies (that lack the Y chromosome) are viable but sterile males (Bridges, 1916). Several studies (Brosseau, 1960; Kennison, 1981; Hazelrigg et al., 1982; Gatti and Pimpinelli, 1983; Gatti and Pimpinelli, 1992) subsequently demonstrated the existence of at least six genetic elements that map on the Y chromosome: four on the long arm (kl-5, kl-3, kl-2 and kl-1) and two on the short one (ks-1 and ks-2), called fertility factors. These loci have a function only in the male germ line, during the development of primary spermatocytes (Marsh and Wieschaus, 1978) (for a review, see Lindsley and Tokuyasu, 1980). More recently, other genetic elements have been mapped on the Y chromosome (Russell and Kaiser, 1993; Gepner and Hays, 1993; Carvalho et al., 2000; Carvalho et al., 2001) indicating that this chromosome has, despite its heterochromatic nature, a complex organization (Fig. 1). Three of the loci originally mapped by Brosseau, namely kl-5, kl-3 and ks-1, have huge physical dimensions, spanning 4000 kb each (Gatti and Pimpinelli, 1983). A large part of their DNA is involved in the formation, inside primary spermatocyte nuclei, of giant lampbrush-like loops that represent the cytological manifestation of their activity (Bonaccorsi et al., 1988). During their growth, Y-loops are loaded with proteins (Bonaccorsi et al., 1988). Some of these proteins have been characterized on the molecular level and are recognized by specific antibodies such as the T53-1 antibody, which specifically recognizes a leucine aminopeptidase associated to the kl-3 loop and the sperm tail (Pisano et al., 1993; Gatti, 1995; Piergentili, 2007), and the S5 antibody, which is directed against a protein associated to nascent RNAs (Saumweber et al., 1980; Risau et al., 1983). Notably, all loops are completely degraded at the beginning of meiotic prophase I. Although transposable elements are an important component of the Y chromosome DNA (Pimpinelli et al., 1995; Berloco et al., 2005), a series of studies (Peacock et al., 1978; Brutlag, 1980) (for a review, see Lohe and Roberts, 1988) demonstrated that ~70% of the Y chromosome DNA is highly repetitive (Fig. 1). In particular, the h3 region, responsible for the formation of the kl-5 loop, contains the AAGAG, AAGAGAG and AAGAC satellites; the h21 region, responsible for the formation of the ks-1 loop, contains the AAGAG and AAGAC satellites; and the h8-h9 region, responsible for the formation of the kl-3 loop, contains the AATAT satellite. At least the AAGAC satellite is abundantly and specifically transcribed inside the kl-5 and ks-1 loops, but these transcripts do not migrate to the cytoplasm and are degraded together with the loops at the beginning of meiosis (Bonaccorsi et al., 1990). To date, no evidence has been provided that the AATAT satellite inside the kl-3 locus is transcribed.

It is known that nucleic acids are flexible molecules and that they can fold in several ways. In particular, the existence of a triple-stranded nucleic acid, usually named 'triplex', was first demonstrated by Felsenfeld and collaborators (Felsenfeld et al., 1957). This conformation is obtained by the insertion of a third strand inside the major groove of a double-stranded (duplex) nucleic acid. Triplexes can contain DNA only, RNA only or a mixture of both (Wells et al., 1988; Roberts and Crothers, 1992; Escude et al., 1993; Booher et al., 1994; Wang et al., 1994; Frank-Kamenetskii and Mirkin, 1995; Spink and Chaires, 1999), and they are stabilized by the Hoogsteen hydrogen bonds. The most stable conformations are those involving DNA alone or an RNA filament entering the major groove of a DNA duplex (Escude et al., 1992). Inside

triplexes, the most stable triads are those involving a protonated cytosine (C$^+$) formed by C$^+$*GC triad and T*AT nucleotides (the asterisks mark the nucleotides on the third strand). Notably, these sequences are over-represented in all eukaryotic genomes (Behe, 1987; Behe, 1995), as well as in eukaryotic viruses (Beasty and Behe, 1988). It is also possible to have triplex formation without homopurine stretches, and these triplexes seem to be as stable as the others at least under certain conditions (Dayn et al., 1992). Although triplexes are well characterized in vitro, their biological significance in living organisms is still under discussion (Zain and Sun, 2003). It has been demonstrated that, upon formation, these structures can frequently downregulate (Cooney et al., 1988; Birg et al., 1990; Faria et al., 2000; Faria et al., 2001) and sometimes upregulate (reviewed in Faria and Giovannangeli, 2001) gene expression, a fact which demonstrates their potential in gene control and suggests their use in gene therapy (Wang et al., 1995; Vasquez et al., 2000) (for a review, see Rogers et al., 2005). The structures are also able to impair DNA polymerization (Dayn et al., 1992), and can influence DNA recombination and repair (Faruqi

Fig. 1. Schematic representation of the Y chromosome of *Drosophila melanogaster*. From top to bottom: cytological localization of satellite DNAs (Bonaccorsi and Lohe, 1991; Lohe et al., 1993), Y chromosome diagram showing N-banding and Hoechst 33258 banding (Pimpinelli et al., 1976; Gatti and Pimpinelli, 1992), cytological localization of transposable elements (Pimpinelli et al., 1995; Berloco et al., 2005), Y chromosome diagram showing the cytological localization and organization of the fertility factors (Gatti and Pimpinelli, 1992), other genetic elements mapping on the Y chromosome (Gatti and Pimpinelli, 1992; Russell and Kaiser, 1993; Gepner and Hays, 1993; Carvalho et al., 2000; Carvalho et al., 2001).

et al., 1996; Wang et al., 1996; Faruqi et al., 2000; Vasquez et al., 2002; Kalish et al., 2005; Raghavan et al., 2005) (for reviews, see Seidman and Glazer, 2003; Chin et al., 2007). Triplexes might also have a role in chromatin organization of both interphase nuclei (Agazie et al., 1996; Ohno et al., 2002) and mitotic chromosomes (Musso et al., 2000). Recently, it has been demonstrated that a triple-stranded pseudo-knot is a conserved and essential element of telomerase RNA, even in humans (Theimer et al., 2005; Shefer et al., 2007). Finally, a number of proteins that are able to bind triplexes have been identified (Kiyama and Camerini-Otero, 1991; Musso et al., 1998; Musso et al., 2000; Nelson et al., 2000), one of which has been isolated in *D. melanogaster* (Jimenez-Garcia et al., 1998). Several techniques have been developed to detect triplexes in vitro (Uddin et al., 1997; Cherny et al., 1998; Rutigliano et al., 1998; Mariappan et al., 1999), but most of these techniques can artificially induce their formation. To date, triplexes can be most reliably detected by using either DNA intercalary substances (Lee et al., 1993; Fang et al., 1994; Moraru-Allen et al., 1997; Shchyolkina and Borisova, 1997) or triplex-directed antibodies. Two antibodies that gave very good results are Jel318, which recognizes T*AT triads, and Jel466, which is more specific for C$^+$*GC triads (Lee et al., 1987; Burkholder et al., 1988; Lee et al., 1989; Agazie et al., 1994; Mol et al., 1994; Agazie et al., 1996). Jel318 intensely decorates the pericentromeric regions of fixed and non-fixed mouse chromosomes, and the G-bands of both human and murine chromosomes (Burkholder et al., 1988; Agazie et al., 1994). The Jel466 staining pattern is complementary to that of Jel318 because it stains R-bands in both human and murine chromosomes (Agazie
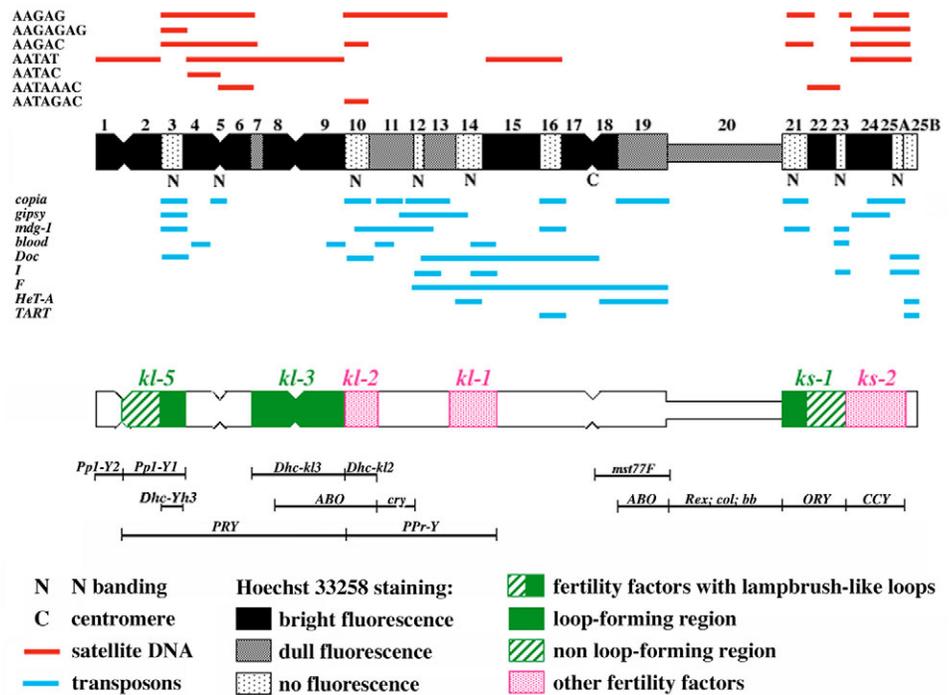
et al., 1994). Taken together, these data support the specificity of Jel318 for AT-rich triplexes, and of Jel466 for GC-rich triplexes. Moreover, they suggest that triplex-forming regions are widespread across the genomes of mammals. It has been demonstrated that triple-stranded nucleic acids are also present inside *D. melanogaster* and *Chironomus tentans* polytene chromosomes (Burkholder et al., 1991). Jel318 strongly stains the bands – and only faintly stains the interbands – of *D. melanogaster* polytene chromosomes. In particular, it intensely stains the 81F band, a transcriptionally silenced region. This band contains the 1672 g/cm^3 satellite DNA consisting of the AATAT and AATATAT sequences. Remarkably, the AATAT satellite DNA is also localized within the kl-3 locus (Fig. 1). Competition experiments demonstrated that Jel318 does not specifically react with this satellite per se (Lee et al., 1987; Agazie et al., 1994), and it has been concluded that the immunostaining exerted by this antibody is due to triplex formation.

In the present paper we used these two antibodies to test the presence of triple stranded nucleic acids inside the lampbrush-like loops formed by the Y chromosome of *D. melanogaster*. We found that two Y-loops, kl-3 and kl-5, are intensely and specifically decorated by Jel318 and Jel466 respectively, indicating that these two structures harbor triple-stranded nucleic acids. Our results suggest that the differential immunostaining exerted by Jel318 and Jel466 is related to the specific molecular organization of each loop. Moreover, we demonstrated the presence of triplexes inside the pseudonucleolus and the clubs Y-loops of *Drosophila hydei*, a species that diverged from *D. melanogaster* at least 58 million years ago. These results suggest that triplexes are a conserved feature of
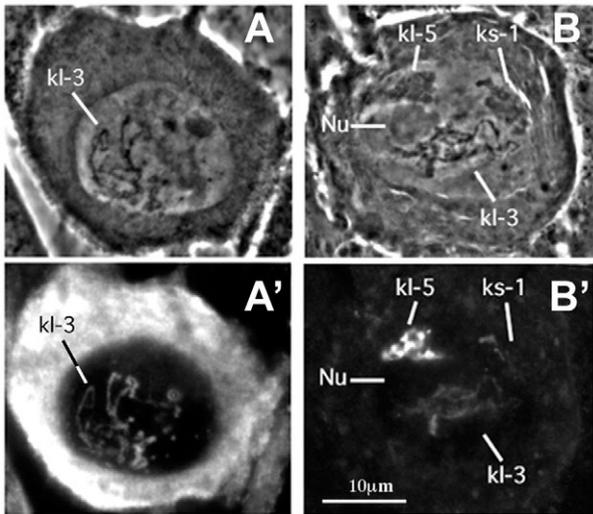
Fig. 2. Cytological characterization of primary spermatocyte nuclei of *D. melanogaster* using Jel318 and Jel466 antibodies. (A,B) Phase contrast images. (A',B') Immunostaining images. (A,A') Jel318 immunostaining of slides pre-treated with trypsin; the antibody specifically recognizes the kl-3 loop but no other intranuclear structure. (B-B') Jel466 immunostaining; the antibody specifically recognizes the kl-5 loop although in some cases also a faint fluorescence on kl-3 loop is present. Note that Jel318 decorates a continuous kl-3 filament whereas Jel466 recognizes a granular matrix inside the kl-5 loop.

Y-loops in drosophilids. Finally, we have found that heat-stress-induced male sterility is coupled to an alteration of triplex immunostaining and, at the same time, to the absence of the high molecular weight polypeptides encoded by the kl-5 and kl-3 fertility factors of *D. melanogaster* (Goldstein et al., 1982).

Results

D. melanogaster Y-loops harbor triple-stranded nucleic acids

Y-loops of *D. melanogaster* are enriched in simple-sequence, satellite DNA (Fig. 1), whose composition is compatible with the formation of triple-stranded nucleic acids. To test the presence of these structures, Jel318 and Jel466 anti-triplex monoclonal antibodies were used to immunostain fixed primary spermatocyte nuclei. The use of a standard fixing procedure permitted us to reveal a specific, intense staining of the kl-5 loop and, occasionally, a faint staining of the kl-3 loop (Fig. 2) with the Jel466 antibody. Jel466 specifically reacts with GC-rich triplexes and its staining is compatible with the DNA composition of the kl-5 loop (Fig. 1) (Bonaccorsi and Lohe, 1991; Lohe et al., 1993). Notably, this antibody does not react with the whole loop, as it happens for the S5 antibody (Bonaccorsi et al., 1988), but produces a granular staining. No staining of the ks-1 loop was observed, although its DNA content is similar to that of kl-5 (Fig. 1). To unambiguously distinguish between the two loops (which are cytologically very similar) we constructed males that carry either the kl-5 or the ks-1 loop. Thus, virgin females of the wild-type *Oregon-R* stock were crossed with *T(X;Y)V24* males (Bonaccorsi et al., 1988). Since the Y chromosome breakpoint of this translocation falls inside the h3-h4 region, the male progeny of this cross bears a deleted Y chromosome missing the h1-h3 region that contains the kl-5 locus. *Oregon-R* females were also mated to *T(X;Y)W19* males (Bonaccorsi et al., 1988). In this case the Y chromosome breakpoint is inside the h20-h21 region and the male progeny of the cross bears a deleted

Y chromosome missing the h21-h25 region, which contains the ks-1 locus. Immunostaining of the two types of males permitted us to demonstrate that Jel466 specifically decorates only the kl-5 loop (data not shown).

Jel318 specifically recognizes AT-rich triplexes but, using the standard fixing procedure, no staining of primary spermatocyte nuclei was detectable with this antibody. However, a weak trypsin pre-treatment of fixed slides before incubation with Jel318 allowed an intense and specific immunostaining of the kl-3 loop (Fig. 2). No staining was ever detected on the other two loops, or the double-stranded DNA of the chromatin clumps that correspond to the premeiotic bivalents. To check the specificity of kl-3 loop immunostaining, X0 males were also analyzed. These males were obtained by crossing *C(1)RM* females with wild-type *Oregon-R* males. In X0 spermatocyte nuclei no triad signal is detectable by immunostaining with either Jel466 or Jel318 under the same experimental conditions described above.

Analysis of DNA fibers reveals that the kl-3 and kl-5 loops have a different organization

To better understand the molecular and cytological organization of the two triplex-forming loops, fibers of DNA were prepared according to Piergentili (Piergentili, 2006). Results are shown in Fig. 3, and it can be seen that the two loops exhibit a different organization. Jel318 specifically stains a thin filament that is barely visible after staining with the DNA dye Hoechst 33258. In some cases, this filament appears quite long and shows no interruptions (Fig. 3A'). In those nuclei with a less squashed chromatin it is possible to detect a more compact kl-3 loop, and the Jel318 antibody decorates a lampbrush-like structure similar to that observed in non-squashed preparations (Fig. 3B'). It is noteworthy that, during fiber preparation, the pre-treatment with trypsin can be omitted without significant differences in the immunostaining (data not shown).

The pattern produced by the Jel466 antibody on the kl-5 loop is different. Similarly to what happens in non-squashed preparations – in which only a part of the loop is stained – the DNA fibers show a discontinuous staining (Fig. 3C'). In many cases it is possible to see aligned dots resembling the filamentous nature of the loop axis. Finally, it is interesting to note that, after a strong squash, Jel466 permits the detection of some small signals within the nuclei of very young primary spermatocytes (Fig. 4). However, before they become unambiguously detectable by S5 immunostaining, it is not possible to assess whether these signals correspond to the primordia of the kl-5 loop or to some other nuclear structure.

To assess whether the loop-associated triplexes are formed by DNA alone, RNA alone, or a mixture of the two, we treated fixed preparations on slides with two different concentrations of a commercial RNase mixture (see Materials and Methods), according to the protocol described by Bonaccorsi and Lohe (Bonaccorsi and Lohe, 1991). Trypsin was used either before or after RNase digestion on intact loops, and fibers were also analyzed in all experimental combinations. In no case was immunostaining using either Jel318 or Jel466 significantly different from that of untreated slides (data not shown).

Triplexes are a conserved feature of Y-loops in drosophilids

It is known that lampbrush-like loops are a conserved feature of primary spermatocytes among drosophilids (Pisano et al., 1993; Piergentili, 2007). Thus, we decided to check whether the presence of triplexes on these structures was also evolutionary conserved. For this purpose, the same antibodies were tested on the male germ
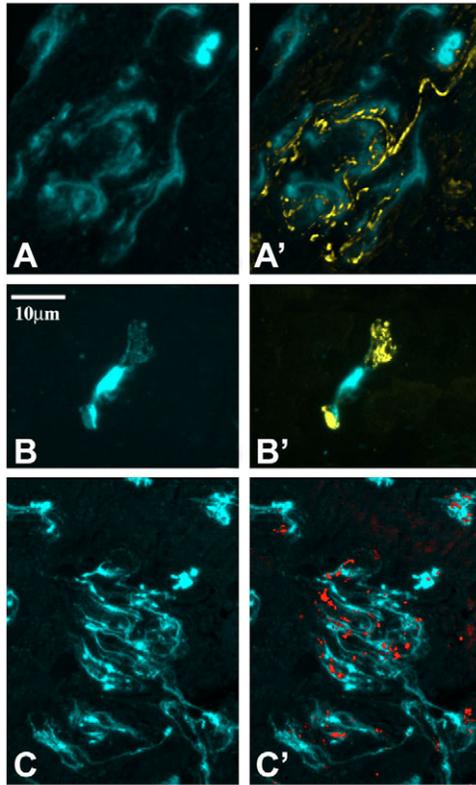
Fig. 3. Analysis of DNA fibers prepared from primary spermatocyte nuclei of *D. melanogaster*. (A-C) Staining with the DNA dye Hoechst 33258. (A'-C') Immunostaining produced by either Jel318 (yellow) or Jel466 (red) merged with the DNA staining (blue). (A') Jel318 specifically recognizes a long, continuous filament barely visible after Hoechst 33258 staining; (B') an almost integer loop occasionally visible in these preparations; image B is slightly overexposed compared with B', to show the DNA nature of the structure decorated by the antibody. (C') Jel466 recognizes a granular, dot-like matrix inside the kl-5 loop (see also Fig. 2); sometimes it is possible to see aligned dots along a DNA axis. Note that none of the antibodies shows cross reactions with the chromatin clumps which are present in these cells.

line of *D. hydei*, a species that diverged from *D. melanogaster* 58 million years ago. Primary spermatocytes of *D. hydei* contain five well-characterized Y-loops, formed by five fertility factors (for a review, see Hackstein and Hochstenbach, 1995). Similarly to *D. melanogaster*, *D. hydei* Y-loops also have huge physical dimensions, but they mainly transcribe middle-repetitive DNA sequences whose nucleotide content is different from that of *D. melanogaster* satellite DNA. Notwithstanding these differences, both Jel318 and Jel466 antibodies are able to intensely decorate at least two *D. hydei* Y-loops, namely the pseudonucleolus and the clubs (Fig. 5). Remarkably, in this case the two antibodies do not show different staining patterns and, in fixed preparations, the trypsin pre-treatment before Jel318 staining can be omitted. Also in this species, the Jel318 and Jel466 immunostaining seems to be specifically restricted to the loops, and no other signal is detected on other intranuclear structures (even with trypsin pre-treatment) or on the chromatin.

Heat stress influences the formation of triplexes and alters the biochemical profile of testis extracts in *D. melanogaster*

To artificially induce an alteration in the triplex structure of the Y-loops, wild-type *Oregon-R* flies were reared at 31 ± 0.5°C for one generation. Under these conditions, male flies become sterile
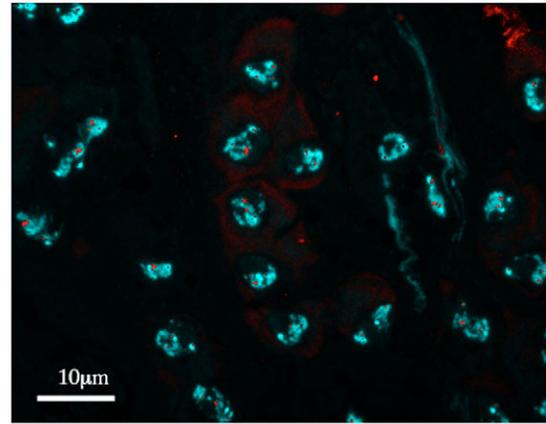

Fig. 4. Jel466-immunostaining of young primary spermatocytes of *D. melanogaster*. Blue, Hoechst 33258 staining; red, Jel466 immunostaining. Young primary spermatocytes were identified according to Cenci et al. (Cenci et al., 1994). All nuclei, prepared according to standard protocols (Piergentili, 2006) show a maximum of four to five small dots that sometimes congregate into large clumps.

(Young and Plough, 1926) and data from the literature demonstrate that sterility is indeed generated in primary spermatocytes (Ayles et al., 1973). Testes prepared from these males and analyzed by phase-contrast microscopy show apparently normal Y-loops. Accordingly, the immunostaining pattern observed with either the S5 or the T53-1 antibody does not seem to be affected (data not shown). However, the immunostaining with both Jel318 and Jel466 failed, even after trypsin pre-treatment, and no signal was ever found in three different sets of experiments (data not shown).

We then evaluated whether heat stress can affect the biochemical profile of the Y-related axonemal dynein heavy chains described by Goldstein and co-workers (Goldstein et al., 1982), by analyzing testis extracts by SDS-PAGE. As shown in Fig. 6A, at least two high-molecular weight bands are missing in the profile, namely those associated with the presence of the kl-3 and kl-5 loci (Goldstein et al., 1982) that are responsible for the formation of the loops decorated by the anti-triplex antibodies. To rule out the possibility that their absence is due to an intrinsic instability of these proteins at high temperature, testes of wild-type males reared at 24 ± 1°C were dissected in physiological solution, quickly transferred to physiological solution at 32 ± 0.5°C for 1 or 2 hours, and then analyzed in the same way. At both timings the electrophoretic profile is indistinguishable from the control (Fig. 6B), indicating that these proteins are stable under these testing conditions.

Discussion

Jel318 and Jel466 are two antibodies that specifically recognize triple-stranded nucleic acids on mammalian chromosomes, without any crossreaction with double-stranded DNA (Burkholder et al., 1988; Agazie et al., 1994). Moreover, they produce a specific staining pattern on both *C. tentans* and *D. melanogaster* polytene chromosomes (Burkholder et al., 1991). The data reported here strongly suggest the presence of triple-stranded nucleic acids also in the male germ line of both *D. melanogaster* and *D. hydei*. In *D. hydei*, both antibodies specifically react with the Y-loops known as pseudonucleolus and clubs (Fig. 5). In *D. melanogaster*, Jel318 and Jel466 specifically react with the kl-3 and the kl-5 loop, respectively (Fig. 2). The difference between these two species may be explained
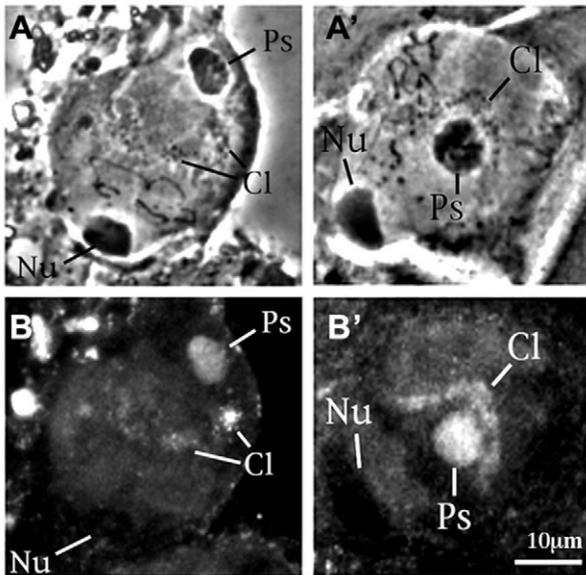
Fig. 5. Cytological characterization of primary spermatocyte nuclei of *Drosophila hydei* using Jel318 and Jel466 antibodies. Both antibodies specifically recognize the pseudonucleolus and clubs loops formed by the Y chromosome of *D. hydei*. Note that Jel318 and Jel466 immunostainings are comparable. No trypsin pre-treatment has been performed on slides incubated with Jel318.

Fig. 6. Electrophoretic analysis of high molecular weight (HMW) ploypeptides expressed in *D. melanogaster* testes. (A) Testis HMW dynein complement from wild-type males reared at either $24\pm1°C$ (lane 1), or at the non-permissive temperature of $31\pm0.5°C$ (lane 2). The HMW dynein bands named as band 3 and band 5 (Goldstein et al., 1982) are clearly visible only in lane 1. (B) HMW electrophoretic pattern of testis extracts from males reared at $31\pm0.5°C$ (lane 1), and extracts of testis dissected from males reared at $24\pm1°C$ and immediately transferred at $32\pm0.5°C$ for either 2 hours (lane 2) or 1 hour (lane 3); note that lanes 2 and 3 exhibit the same electrophoretic pattern as controls (panel A, lane 1), indicating that bands 3 and 5 are stable under these experimental conditions.

by the fact that the middle repetitive sequences of the *D. hydei* Y-loops are not particularly enriched in either CG or AT doublets, which suggests that triplexes of this species are composed of a mixture of different triads. Instead, in *D. melanogaster* the kl-5 loop is enriched in GC doublets, wheras the kl-3 loop is enriched in AT doublets (Fig. 1), possibly indicating a different organization of the triplexes. It is interesting to note that the ks-1 locus contains and abundantly transcribes the same satellite DNAs that are present inside the kl-5 locus (Fig. 1); moreover, both loops are recognized by the S5 antibody (Saumweber et al., 1980). Nonetheless, under our experimental conditions the ks-1 loop was never stained when using Jel466. Thus, our data confirm the specificity of this antibody for the triplex structure and not for a particular DNA sequence (Agazie et al., 1994).

It is known that at least the AAGAC satellite DNA associated to the kl-5 locus is abundantly transcribed, and that the resulting RNA does not migrate to the cytoplasm, being degraded prior to meiotic prophase I (Bonaccorsi et al., 1990). However, no evidence has been provided to date about the transcription of the kl-3-associated AATAT satellite, although some open reading frames have been mapped inside this Y chromosome region (Fig. 1). It is known that the best prerequisite for triplex formation is the presence of a homopurine stretch of nucleotides on one filament and a corresponding homopyrimidine stretch on the complementary strand, but this does not occur in this case. Nevertheless, it should be taken into account that an AATAT-associated triplex has been already described by Dayn and collaborators (Dayn et al., 1992), and that the AATAT- and AATATAT-rich region 81F of polytene chromosomes of *D. melanogaster* is intensely stained by Jel318 (Burkholder et al., 1991), indicating that this satellite DNA may indeed form triple-stranded nucleic acids. We believe that, although not 'perfect', the organization and nucleotide content of the kl-3 locus is sufficient to force the formation of triplex structures, even
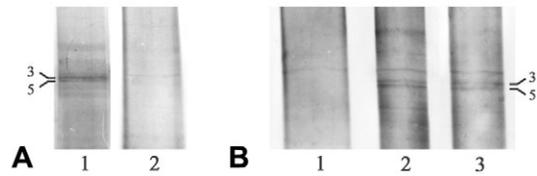
inside the kl-3 loop. It is possible to hypothesize that the torsional stress induced by the presence of an open chromatin conformation and the contemporary absence of free loop ends (Bonaccorsi et al., 1988) supplies the necessary energy to establish such structure. The mechanism could be similar to that used by other non B-DNA structures such as Z-DNA, even in the absence of a homopurine sequence. RNase-treated slides did not show significant differences with respect to non-treated slides in the immunostaining pattern produced by either Jel318 or Jel466. Although we cannot completely rule out the possibility that RNase treatment is inefficient under our experimental conditions, these data suggest that, in Y-loops RNA is not part of the triplex structure, and both kl-3 and kl-5 harbor intramolecular DNA triplexes.

Since the AT content and the GC content of the AAGAG and AAGAGAG satellites are comparable (Fig. 1), it is rather surprising that the kl-5 loop is not stained by either of the anti-triplex antibodies. However, it is known that, at the molecular level, the two antibodies recognize the Hoogsteen triads differently (Agazie et al., 1994). We believe that the T*AT triads inside the kl-5 loop triplex, because of the simultaneous presence of the C^+*GC triads recognized by Jel466, have a geometry that is incompatible with the Jel318 antibody recognition. A difference between the kl-5- and kl-3-associated triplexes is suggested also by the pattern of the immunostaining signals produced by the antibodies inside primary spermatocyte nuclei. Jel318 decorates a long, continuous filament resembling the appearance of the kl-3 loop immunostained by T53-1 (Pisano et al., 1993). This suggests that probably the entire DNA axis of this loop is involved in the formation of the triplex structure. However, immunostaining obtained with the Jel466 antibody on the kl-5 loop consists of a set of discrete dots that, in fiber preparations, appear aligned along a DNA filament. This suggests that the triplex-forming regions inside the kl-5 loop are either discontinuous, or that only a part of the triplex structure is accessible to Jel466. The former possibility might be supported by the evidence that in *D. melanogaster* only the AAGAGAG satellite has been tested for triplex formation (Horn et al., 2004; Paris et al., 2007); no data are available to date for the other two sequences. It is interesting to note that this satellite is not present inside the ks-1 locus (Fig. 1) and, under our experimental conditions, the corresponding Y-loop did not stain using Jel466. The latter possibility is indirectly supported by the fact that Jel318 staining can be obtained only after pre-treating the slides with trypsin or after the strong squashing that allows fiber visualization. These data

suggest that Y-loop-associated triplexes are associated to proteins, probably triplex-binding units that can be removed either by a weak proteolytic digestion or by a mechanical shock, as it happens during fiber preparation.

In both *D. melanogaster* and *C. tentans* polytene chromosomes there is no triplex immunostaining associated to intensely transcribed puffs, suggesting that triplexes are present in transcriptionally inactive regions of the genome (Burkholder et al., 1991). This association is also supported by a large number of studies on various systems indicating that triplex formation inside the promoter region usually downregulates gene expression (reviewed by Faria and Giovannangeli, 2001). However, it is unlikely that this is the function of the triplexes associated to the lampbrush-like loops of primary spermatocytes, because both the kl-5 and *D. hydei* Y-loops are transcriptionally active (Bonaccorsi et al., 1990; Hackstein and Hochstenbach, 1995). We hypothesize that these triplexes are able to mediate the interaction between DNA-specific regions and some proteins that might recognize triplex structure as a specific binding site. Indeed, at least one triplex-binding protein has been already identified in *D. melanogaster* (Jimenez-Garcia et al., 1998).

It has long been known that primary spermatocytes are sensitive to both cold and hot stresses (Young and Plough, 1926; Sanders and Ayles, 1970; David et al., 1971; Cohet, 1973; Ayles et al., 1973; Suchowersky et al., 1974). Since the most typical feature of these cells is the presence of lampbrush-like loops, we checked whether heat stress can affect these intranuclear structures. Although apparently normal, Y-loops of flies that have been reared at 31±0.5°C showed no reaction using either the Jel318 or the Jel466 antibody. Biochemical analysis of testis extracts from flies reared at 31±0.5°C showed that at least two of the putative dynein heavy chains described by Goldstein and co-workers (Goldstein et al., 1982) were missing. Remarkably, these polypeptides correspond to those encoded by the triplex-containing kl-3 and kl-5 loops. This biochemical phenotype resembles that exhibited by some autosomal mutants (Piergentili et al., 2004), in which the kl-3 loop is absent or strongly reduced. However, after heat stress, both the kl-3 and the kl-5 loops are present and still able to bind at least the proteins recognized by the S5 and T53-1 antibodies. The easiest explanation for these observations is that, analogous to what has been observed with autosomal mutations, the two proteins are produced but they are unstable and possibly rapidly degraded. Taken together, these data suggest that triplexes have a role in protein stabilization. They might serve as a docking, storing and/or modification site of proteins synthesized before meiosis, but exerting their function in post-meiotic stages. If this hypothesis will be further supported, Y-loops of drosophilids will be the first example of triplexes whose role is not only functional but also structural, i.e. protein docking. Another possibility, although restricted to the kl-5 loop, is that triplexes are involved in the splicing of the extremely long introns among which the coding sequences of some genes are interspersed (Fig. 1). Indeed, it has been demonstrated that a polypurine stretch with alternating A and G can stimulate (Tanaka et al., 1994) or inhibit (Carstens et al., 2000) the splicing of the surrounding intron(s). Moreover, a triplex structure has been described inside an intron splicing intermediate (Adams et al., 2004). Of course, it is still possible that triplexes have both roles in the *D. melanogaster* male germ line, further supporting the differences between the two loops in both content and structure (Bonaccorsi and Lohe, 1991; Lohe et al., 1993) (our data): a protein-docking role on the kl-3 loop (where no RNA transcription has been demonstrated to date) and an intron-splicing role on the kl-5 loop.

Materials and Methods

Drosophila strains
Drosophila melanogaster [*Oregon-R*, *C(1)RM, T(X;Y)V24* and *T(X;Y)W19* lines] and *Drosophila hydei* stocks were kindly provided by M. Gatti and S. Bonaccorsi at the University of Rome 'Sapienza'; the karyotype of the *D. melanogaster* lines have been extensively described by Lindsley and Zimm (Lindsley and Zimm, 1992). All lines were reared at 24±1°C using standard cornmeal medium. When appropriate, the wild-type *Oregon-R* line was mated at 24±1°C for 2 days in glass bottles and then transferred for one generation in thermostat at 31±0.5°C.

Testis immunofluorescence
Larval, pupal or adult testes were dissected and fixed according to Bonaccorsi et al. (Bonaccorsi et al., 1988) and Pisano et al. (Pisano et al., 1993). After fixation, slides were rinsed twice in Dulbecco's PBS modified formula for 5 minutes and incubated for 1 hour in a humid chamber at room temperature with one of the following: 20 µl of the T53-1 antiserum (Pisano et al., 1993) diluted 1:10 in PBS, 20 µl of the S5 monoclonal antibody (Saumweber et al., 1980) diluted 1:20 in PBS, 10 µl of Jel466 monoclonal antibody (Agazie et al., 1994) diluted 1:10 in PBS or 10 µl of Jel318 monoclonal antibody (Lee et al., 1987) diluted 1:10 in PBS. As for the last antibody, when preparing intact loops for *Drosophila melanogaster*, a pre-treatment of each slide with trypsin (10 µl at 25%) for 60-90 seconds was necessary before antibody incubation; after the pre-treatment, slides were rapidly washed in PBS and subsequently transferred for 3 minutes in clean PBS, to avoid excessive trypsin contact. Then all slides were washed twice in PBS for 5 minutes and incubated 1 hour with the secondary antibody, a sheep anti-mouse IgG conjugated with fluorescein (FLUOS, Boehringer), diluted 1:20 in PBS. Slides were finally air dried and mounted in PBS containing 0.5 µg/ml Hoechst 33258 dye. Analysis of DNA fibers was performed using a standard protocol (Piergentili, 2006). The entire preparation was performed the same day, without interruptions. In another set of experiments, all slides prepared according to the above methods were also treated with RNase to test the presence of RNA inside triplexes. According to published protocols (Bonaccorsi and Lohe, 1991), 20 µl of a DNase-free RNase mixture (Roche) (2 µg/ml or 10 µg/ml in 2×SSC) were put onto the preparations and incubated for 30 minutes at room temperature in a humid chamber. Subsequently, slides were washed twice in 2×SSC and immunostaining was performed. The RNase treatment was performed either before or after the trypsin digestion in both Jel318 and Jel466 immunostained slides, for both standard and fiber preparations. Cell stages were identified as described by Cenci and co-workers (Cenci et al., 1994).

Light mcroscopy
Microscope analysis and pictures were made using a Zeiss III RS photomicroscope equipped with an HBO fluorescent light (100 Watts), or with a Zeiss Axioplan photomicroscope equipped with an HBO fluorescent light (50 Watts). The Zeiss filter combination 09 was used for immunostained preparations with FLUOS-conjugated secondary antibodies, and Zeiss filter combination 01 for staining using Hoechst dye 33258. Pictures at the Zeiss Axioplan microscope were taken using a CCD camera from Photometrics and saved using IP Lab Spectrum software. Pseudocoloration of micrographs was performed using the Adobe Photoshop® software.

Electrophoretic analysis of testis protein from heat-induced sterile males
Testes from males reared at 31±0.5°C were dissected and immediately denatured; their high molecular weight protein complement was then analyzed by electrophoresis on 3-5% SDS-polyacrylamide gels, according to Laemmli (Laemmli, 1970), using a constant amperage of 7 mA in the stacking gel and of 15 mA in the separation gel. After electrophoresis, gels were silver stained by the method of Wray et al. (Wray et al., 1981).

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