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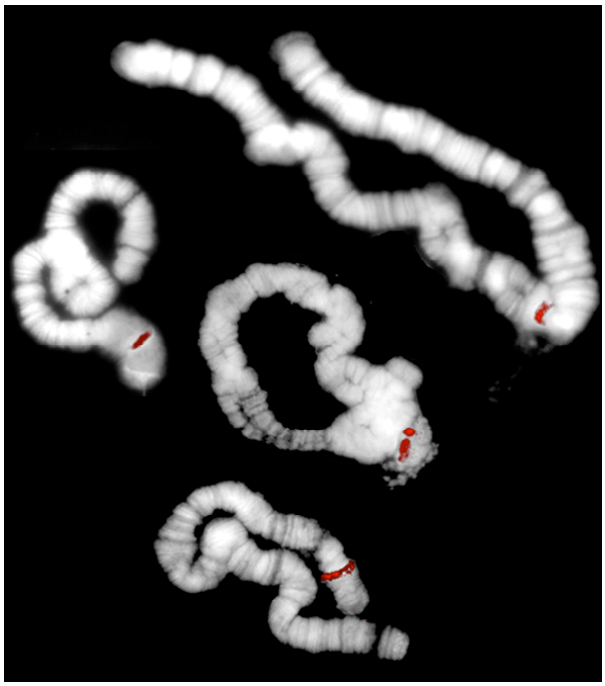
Análisis celular y molecular de la heterocromatina en *Sciara*



Mª del Carmen Escribá Pérez

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Análisis celular y molecular de la heterocromatina en *Sciara*

Memoria presentada por Mª del CARMEN ESCRIBÁ PÉREZ para optar al grado de
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VºBº Autor

Mª del Carmen Escribá Pérez

VºBº Directores

Dr. Alfredo Villasante Atienza
Centro de Biología Molecular Severo Ochoa
Universidad Autónoma de Madrid, CSIC

Dra. Clara Goday Baylina
Centro de Investigaciones Biológicas
CSIC

VºBº Tutor

Dr. Julio Sánchez Rufas
Departamento de Biología
Facultad de Ciencias.
Universidad Autónoma de Madrid

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Abreviaturas

CE: Elemento controlador (“*Controlling Element”*)
Cromosomas L: Cromosomas limitados a la línea germinal (*Germline Limited)*
DNA: Ácido desoxiribonucléico, ADN (*“Deoxyibonucleic Acid”*)
LM: (*“Land Mark”*)
LTR: Repetición terminal larga (“*Long Terminal Repeat”*)
ORF: Marco abierto de lectura (*Open Reading Frame*)
PGC: Células primordio-germinales (*“Primordial Germ Cells”*)
rDNA: DNA ribosómico
RNA: Ácido ribonucléico, ARN (*“Ribonucleic Acid”*)
siRNA: (*“Small Interfering RNA”*)
RTE: Retrotransposón (*“Retrotransposable Element*)
X_m: Cromosoma X materno
X_p: Cromosoma X paterno

ÍNDICE

Resumen

RESUMEN

El díptero *Sciara*, y en particular las especies *S. coprophila* y *S. ocellaris*, constituyen un buen modelo experimental para analizar alteraciones programadas de la segregación cromosómica durante el desarrollo. En ambos organismos se produce la eliminación selectiva de uno o dos cromosomas X paternos durante las divisiones somáticas del embrión y la no-disyunción del cromosoma X materno durante la meiosis II masculina. Ambos procesos implican alteraciones importantes en la función centromérica y están regulados por un mismo *locus* (*locus CE*) cuyas secuencias específicas de DNA se desconocen todavía. En *S. coprophila* se ha determinado que el *locus CE* reside en la heterocromatina adyacente al centrómero del cromosoma X.

En la primera parte de este trabajo de Tesis Doctoral se ha procedido a abordar, por primera vez, el estudio citológico y molecular de la heterocromatina en los cromosomas de *S. coprophila.* Para ello, se ha llevado a cabo la microdisección de la región centromérica del cromosoma politénico X y la posterior amplificación y clonaje del DNA obtenido. La localización cromosómica de los clones seleccionados se ha realizado mediante experimentos de hibridación "*in situ"* en cromosomas politénicos, mitóticos y meióticos. Este trabajo ha permitido la identificación y caracterización de dos repeticiones de la región pericentromérica del cromosoma X, un retrotransposón pericentromérico (*RTE*) y un satélite centromérico rico en ATs.

En la segunda parte del trabajo se han explorado las modificaciones covalentes de las histonas relacionadas con la ocurrencia del fenómeno de la no-disyunción del cromosoma X materno durante la meiosis masculina. Para ello, en espermatocitos de *S. coprophila* y *S. ocellaris,* se ha estudiado mediante inmunofluorescencia los patrones de fosforilación de la histona H3 en los residuos de serina 10/28 y treonina 3/11. Los resultados han permitido demostrar que durante la meiosis II masculina el cromosoma X

materno difiere del resto de cromosomas, ya que su región centromérica no está fosforilada para la histona H3. Así, se ha relacionado por primera vez, en las dos especies, la falta de fosforilación de la histona H3 en la región centromérica del cromosoma X con su inactivación funcional.

Por último, se ha iniciado la caracterización de secuencias teloméricas en *Sciara coprophila.* Para ello, se ha microdiseccionado y microclonado la región telomérica 1A del cromosoma IV. El análisis de las secuencias obtenidas ha permitido caracterizar una secuencia telomérica específica de este telómero.

Introducción

INTRODUCCIÓN

La eliminación programada de cromosomas enteros, o bien de fragmentos cromosómicos, constituye un fenómeno biológico notable que ocurre en un considerable número de organismos pertenecientes a diferentes grupos taxonómicos. Los ejemplos más conocidos se han descrito en ciliados, nematodos, crustáceos, insectos y en algunos vertebrados (revisión en White, 1973; Goday y Pimpinelli, 1993). Este peculiar fenómeno tiene lugar durante el desarrollo, y a menudo ocurre durante la separación de las líneas germinal y somática. La exclusión programada de cromatina se puede considerar como una forma extrema de inactivación génica, funcionalmente comparable con la inactivación de la cromatina por "heterocromatinización", como es el caso de la inactivación selectiva del cromosoma X paterno en embriones de mamíferos (Lyon, 1961).

El díptero *Sciara* (Nematocera, F. Sciaridae), constituye un modelo clásico de eliminación de cromosomas enteros en diferentes tejidos durante el desarrollo (Metz y Moses, 1926; Dubois, 1933; Gerbi, 1986; Goday y Esteban, 2001). En *Sciara*, además, se usó por primera vez el término "impronta genómica" al descubrirse que los cromosomas que se eliminan son exclusivamente de origen paterno (Crouse, 1960). Actualmente, se sabe que cuando el comportamiento diferencial de un cromosoma (o la expresión diferencial de un gen o grupo de genes) depende de su origen parental, esto constituye una forma epigenética de regulación presente en el desarrollo de numerosos organismos.

La eliminación de cromosomas en *Sciara* implica modificaciones en procesos biológicos básicos como son la segregación cromosómica en mitosis y en meiosis (Gerbi, 1986; Goday y Esteban, 2001). Por ello, desde los estudios clásicos se ha considerado que estos organismos son un modelo óptimo para analizar el comportamiento cromosómico. Todos los ciáridos presentan un ciclo cromosómico prácticamente idéntico, siendo *Sciara coprophila* y *Sciara ocellaris* las especies más estudiadas.

En cuanto a la organización molecular del genoma de *Sciara* se han caracterizado tanto en *S. coprophila* como en *S. ocellaris*, el gen *Sex-lethal* (Ruiz *et al.*, 2003; Serna *et al.*, 2004) y el gen *transformer (*Martin *et al., 2011)*, y en *S. coprophila* los genes ribosomales (Gerbi, 1971; Gerbi y Crouse, 1976) y los genes *Scohet1* y *Scohet2* (Greciano *et al.*, 2009). El complemento cromosómico regular de *S. coprophila* y *S.*

ocellaris está constituido por tres pares de autosomas (cromosomas II, III y IV), y uno o dos cromosomas X (Fig. 1). Los cromosomas II, III, y X son acrocéntricos, mientras que el cromosoma IV es metacéntrico. (Rieffel y Crouse, 1966; Amabis *et al.*, 1979; Goday y Ruiz, 2005; Greciano *et al.*, 2009). Es importante destacar que gran parte de los estudios citológicos clásicos realizados en *Sciara,* se han llevado a cabo en cromosomas politénicos (Fig. 1); a diferencia de otros dípteros como *Drosophila*, en *Sciara* dichos cromosomas no forman un cromocentro, lo que permite visualizarlos y analizarlos separadamente. El uso de tinciones con fluorocromos y las técnicas de bandeo C permitieron obtener los mapas citológicos de todos los cromosomas del complemento cromosómico de *S. coprophila.* Además, mediante el análisis de translocaciones recíprocas se pudo determinar la localización de los centrómeros (flechas en la figura 1) (Crouse, 1943; Gabrusewycz-Garcia, 1964; Crouse, 1977).

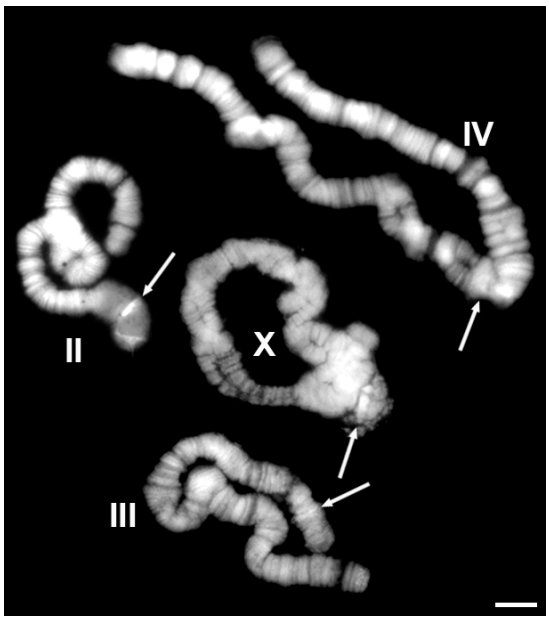

Figura 1. Cromosomas politénicos de las glándulas salivales de *S. coprophila*. Tinción de cromatina con DAPI. Las flechas señalan la posición del centrómero en cada cromosoma (**II, III, IV** y **X**). Barra, 10 μm.

En *S. coprophila* además de los cromosomas del complemento regular, existen cromosomas accesorios denominados "cromosomas L" que son exclusivos de la línea germinal y cuyo número es variable (Fig. 2). Los cromosomas L, de función

desconocida, son altamente heterocromáticos y su DNA está enriquecido en residuos 5-metilcitosina (Greciano *et al.*, 2009).

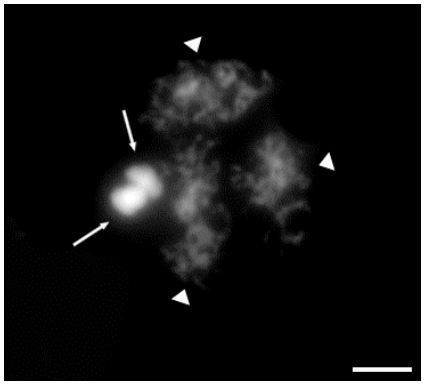

Figura 2. Núcleo interfásico premeiótico masculino de *S. coprophila*. Tinción de cromatina con DAPI. Los cromosomas L (flechas) se tiñen mas intensamente debido a su alto grado de condensación con respecto al complemento cromosómico regular (cabezas de flecha). Barra, 10 μm.

Ciclo cromosómico de *Sciara*

El ciclo cromosómico de *S. coprophila* y *S. ocellaris* es atípico y extremadamente complejo (Figura 3). Durante las primeras divisiones embrionarias se eliminan uno o dos cromosomas X paternos (X_p) dependiendo del sexo del embrión, en hembras y machos respectivamente. Asimismo, cuando existen cromosomas L, como es el caso de *S. coprophila*, se produce la eliminación de todos ellos del soma embrionario en las divisiones nucleares que preceden a la eliminación de los cromosomas X paternos. En etapas embrionarias mas avanzadas, ocurre la eliminación de un solo cromosoma X_p de todos los núcleos pregerminales (células primordio-germinales o PGC). Por otra parte, durante la última etapa larvaria, en la que tiene lugar la meiosis, se produce una modificación en la meiosis masculina dado que durante la meiosis I todo el complemento cromosómico paterno es eliminado del espermatocito. Este evento, altamente anómalo, solo tiene lugar en la línea germinal masculina mientras que en la femenina la meiosis es ortodoxa. Otra característica notable de la meiosis masculina es la no-disyunción del cromosoma X materno (X_m) durante la meiosis II. Como consecuencia, el núcleo del espermatozoide contiene dos cromosomas X_m idénticos o isocromosomas. Dichos cromosomas serán reconocidos en el siguiente ciclo

cromosómico como paternos (Metz y Moses, 1926), lo que indica que la impronta genómica revierte en cada ciclo cromosómico (Rieffel y Crouse, 1966; Gerbi, 1986).

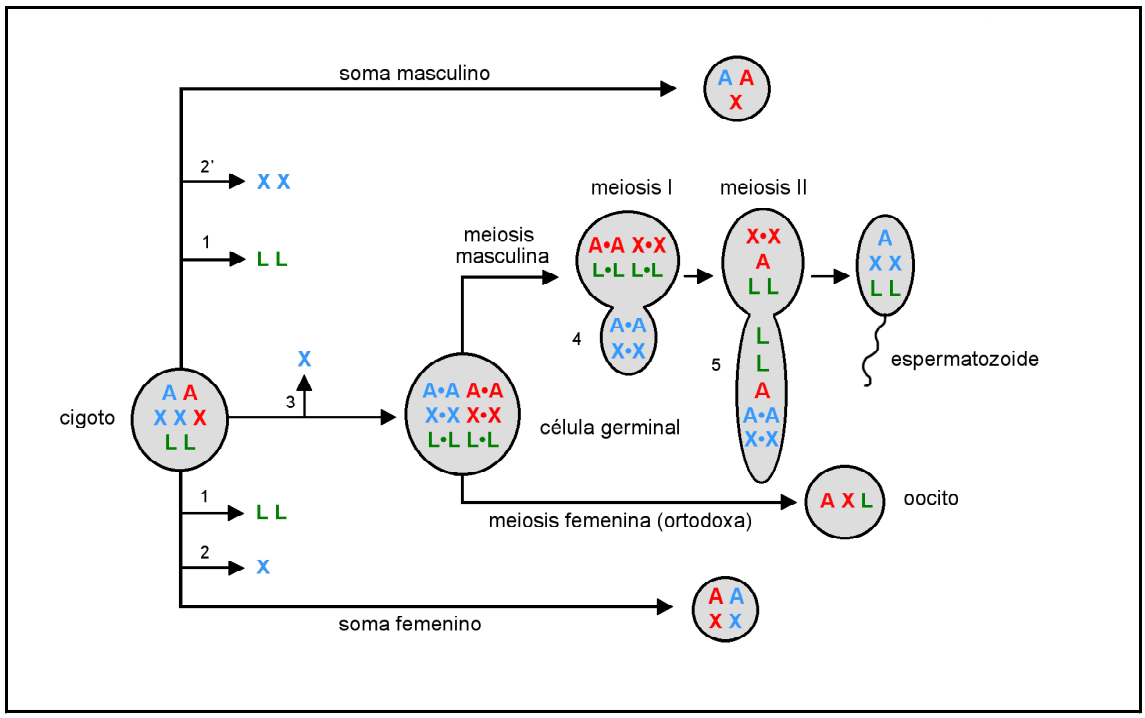

Figura 3. **Diagrama del ciclo cromosómico de *Sciara coprophila*. A** representa autosomas, **X** cromosomas X, y **L** cromosomas L El color rojo indica cromosomas maternos y el azul paternos. Los números indican los eventos de eliminación cromosómica. El zigoto contiene tres pares de autosomas y tres cromosomas X. Durante las divisiones embrionarias 5ª y 6ª, se eliminan todos los cromosomas L de las células somáticas (1). Durante las divisiones 7ª a 9ª, en todas las células somáticas se elimina un cromosoma X_p en las hembras (2) y dos cromosomas X_p en los machos (2'). En etapas embrionarias posteriores, en las células germinales se elimina un cromosoma X paterno (3). Por ello, todos los núcleos germinales pasan a contener solamente un cromosoma X_p, y un cromosoma X_m (nótese que en las células germinales se representan las cromátidas hermanas de los cromosomas separadas por un punto). La meiosis femenina es ortodoxa, pero en la masculina todo el complemento cromosómico paterno es eliminado durante la meiosis I en una vesícula citoplásmica (4). En la meiosis II masculina, los autosomas maternos y cromosomas L segregan normalmente, mientras que el cromosoma X materno no separa las cromátidas y ambas son incluidas en el núcleo del espermatozoide. El grupo de cromátidas maternas que carece de cromosoma X es incluido en la vesícula citoplásmica y es eliminado junto a los cromosomas paternos (5). Nótese que en el núcleo del espermatozoide el origen materno de los cromosomas (rojo) revierte a paterno (azul), ya que así serán reconocidos tras la fertilización.

Eliminación de cromosomas X_p del soma embrionario

Durante las primeras divisiones embrionarias, y en todas las células somáticas, se eliminan uno o dos cromosomas X_p en machos y hembras, respectivamente (Dubois, 1932; Dubois, 1933). El fenotipo citológico de la eliminación somática del cromosoma X_p se denominó "retardo anafásico" (Fig. 4) (Metz, 1926). Los cromosomas X_p comienzan normalmente el movimiento anafásico hacia los polos pero son incapaces de completarlo y, por lo tanto, no son incluidos en el núcleo de las células hijas (Dubois, 1932; Dubois, 1933). Los mecanismos moleculares involucrados en este proceso son todavía desconocidos, no obstante, se ha propuesto que modificaciones en la función del centrómero, así como en el proceso de separación de las cromátidas hermanas, podrían estar directamente implicados en este tipo de eliminación cromosómica (Dubois, 1932; Gerbi, 1986; de Saint-Phalle y Sullivan, 1996; Goday y Esteban, 2001). En *S. coprophila*, los cromosomas L, limitados a la línea germinal, son excluidos del soma embrionario mediante un proceso idéntico al descrito para los cromosomas X_p (Rieffel y Crouse, 1966). Se ha demostrado que el número de cromosomas X_p eliminados está regulado por factores citoplásmicos maternos distribuidos en el huevo (Dubois, 1932; Dubois, 1933; Gerbi, 1986; Perondini *et al.*, 1986). La naturaleza de dichos factores no se conoce todavía, aunque se sabe que se generan durante la oogénesis (Nigro, 1995; Perondini, 1998).

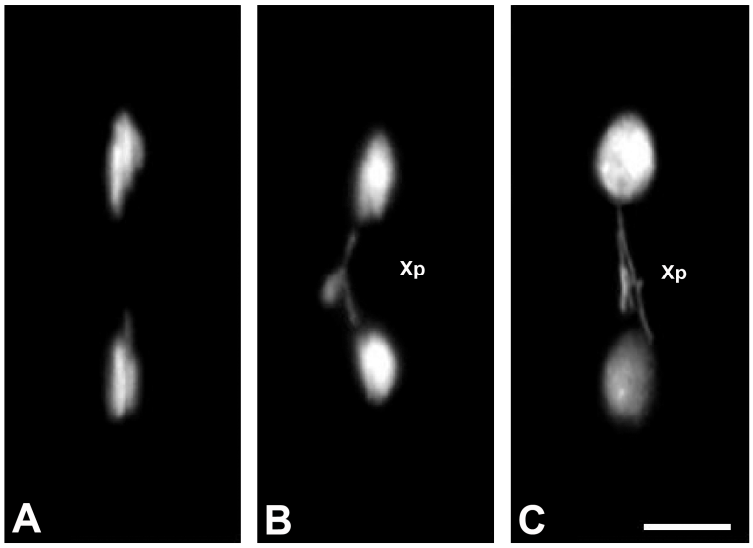

Figura 4. Eliminación somática de cromosomas X paternos de *S. ocellaris*. Mitosis somáticas en embriones tempranos teñidos con DAPI; **Xp**, cromosoma X paterno. (A) Anafase pre-eliminación. (B) Anafase con un cromosoma Xp en "retardo anafásico". (C) Telofase con dos cromosomas Xp eliminados. Barra, 10 μm.

Meiosis masculina

En *Sciara*, durante la meiosis masculina, el comportamiento de los cromosomas es altamente inusual debido a que todo el complemento cromosómico paterno es eliminado, por lo que sólo los cromosomas de origen materno forman parte del núcleo del espermatozoide. Otra característica excepcional de la meiosis masculina es la no disyunción del cromosoma X_m, hecho que determina la constitución 3X típica del zigoto (Gerbi, 1986; Goday y Esteban, 2001). Desde los estudios clásicos del ciclo cromosómico en *S. ocellaris* y *S. coprophila,* los mecanismos responsables de la eliminación del complemento cromosómico paterno en la meiosis I y la no disyunción del cromosoma X_m en la meiosis II han sido objeto de diversos estudios (revisión en (Gerbi, 1986; Goday y Esteban, 2001). En la Figura 5 se resumen los procesos más relevantes de la meiosis masculina comunes a todos los ciáridos. Durante la primera división meiótica no hay apareamiento de cromosomas homólogos durante la profase y los cromosomas no se alinean en una placa metafásica (Metz, 1925; Metz, 1926a; Metz, 1926b; Fuge, 1994). En lugar de ello, los cromosomas pasan directamente de prometafase I (Fig. 5A) a un estadio similar a una anafase I (Fig. 5B). En este proceso interviene un huso monopolar generado por un único complejo polar conteniendo "centriolos gigantes" (Fig. 5A-B) y gran cantidad de material pericentriolar del cual irradian numerosos microtúbulos (Kubai, 1982; Fuge, 1994; Esteban *et al.*, 1997). En la peculiar anafase I, el huso monopolar dirige la segregación de los cromosomas maternos hacia el polo del huso, mientras que los cromosomas paternos se mueven en dirección opuesta hacia una vesícula citoplásmica que será posteriormente escindida (Metz, 1925; Smith-Stocking, 1936). Es importante destacar que según los datos citológicos y ultraestructurales, desde los estadios iniciales del desarrollo hasta el inicio de la meiosis los dos complementos cromosómicos parentales ocupan compartimentos celulares diferentes en el núcleo germinal (Rieffel y Crouse, 1966; Kubai, 1982; Kubai, 1987; Goday y Esteban, 2001; Goday y Ruiz, 2002). El hecho de que los cromosomas de origen materno estén separados precozmente respecto de los paternos se considera esencial en la determinación de qué conjunto cromosómico es eliminado durante la meiosis I masculina (Kubai, 1987; Goday y Esteban, 2001).

La característica más significativa de la meiosis II masculina es el comportamiento cinético diferencial del cromosoma X_m respecto a los autosomas maternos (Metz, 1925; Crouse, 1943; Esteban *et al.*, 1997). Durante la metafase II (Fig.

5C), mientras los autosomas maternos forman la típica placa metafásica, el cromosoma X_m permanece anclado al huso de la primera división meiótica. Es importante señalar que los microtúbulos que irradian del único polo del huso interaccionan con el centrómero del cromosoma X_m y mantienen la no-disyunción de sus cromátidas a lo largo de toda la meiosis (Esteban *et al.*, 1997). Finalmente, en la anafase II (Fig. 5D-E) las cromátidas de los autosomas segregan normalmente por medio de un segundo huso bipolar (Metz, 1926; Abbott *et al.*, 1981; Esteban *et al.*, 1997). En el caso de *S. coprophila*, los cromosomas L segregan junto a los cromosomas de origen materno durante la meiosis masculina con independencia de su origen parental (Metz, 1938; Crouse *et al.*, 1971).

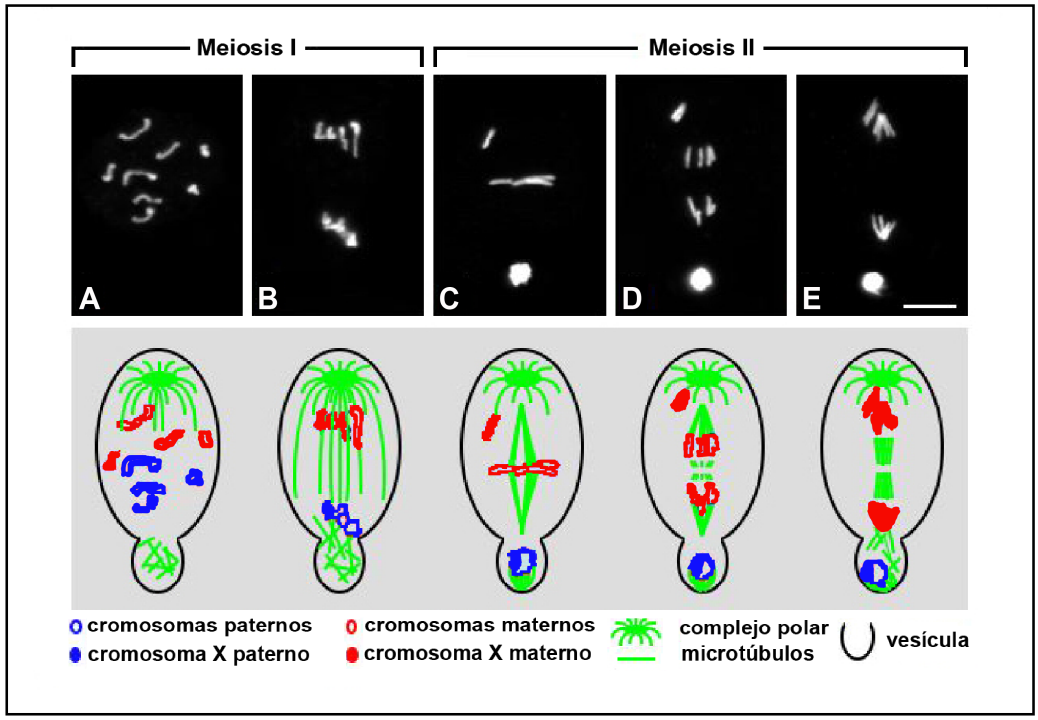

Figura 5. Meiosis masculina en *Sciara ocellaris.* En la parte supeior (A-E), cromosomas teñidos con DAPI. (A) profase I, (B) anafase I, (C) metafase II, (D,E) anafase II. En la parte inferior, diagrama de las imágenes mostradas arriba ilustrando las interacciones de los cromosomas con los microtúbulos. Barra, 10 μm.

Los datos existentes sobre la meiosis masculina en *Sciara* han llevado a proponer un modelo que relaciona la compartimentación intranuclear de los cromosomas maternos y paternos, la eliminación cromosómica y determinadas modificaciones de histonas (Greciano y Goday, 2006). De acuerdo con este modelo, en *S. ocellaris* se

demostró la existencia de diferencias en la acetilación/metilación de las histonas H3 y H4 entre los cromosomas maternos/paternos durante la meiosis I masculina (Goday y Ruiz, 2002; Greciano y Goday, 2006). Por otra parte es de interés destacar aquí, que hasta el momento se ha aceptado que el fenómeno de no-disyunción meiótica del cromosoma X_m es debido a modificaciones en la función centromérica (Gerbi, 1986; Goday y Esteban, 2001). Se desconoce si modificaciones epigenéticas de la cromatina están directamente involucradas en dicho proceso; en particular, modificaciones conservadas de histonas, como es la fosforilación de H3 esencial en mitosis/meiosis en organismos eucariotas (Gurley *et al*., 1978; Van Hooser *et al*., 1998; Goto *et al*., 1999; Wei *et al*., 1999; Giet y Glover, 2001).

El elemento controlador o *"locus CE"*

Mediante el análisis de translocaciones recíprocas entre el cromosoma X y los autosomas en *S. coprophila* (Fig. 6A), se observó que la integridad del cromosoma X no es requisito indispensable para que tenga lugar la no-disyunción de este cromosoma durante la meiosis II masculina. En concreto, para que ocurra este fenómeno solamente la región heterocromática próxima al centrómero del cromosoma X es estrictamente necesaria (Crouse, 1943). Esta observación fue posteriormente ampliada tras el estudio de varias translocaciones adicionales, entre ellas la translocación T1 (Fig. 6B) en la que el cromosoma X no contiene dichos bloques heterocromáticos al haber sido translocados al cromosoma II (Crouse, 1960). En dicha translocación se observa que durante la meiosis II el cromosoma X^{II} es ahora capaz de alinearse en la placa metafásica II y segregar normalmente (Crouse, 1960). Por el contrario, el cromosoma II^{x} portador de la heterocromatina proximal del cromosoma X_m se comporta como un cromosoma X_m típico, que como se ha mencionado, no forma parte de la placa metafásica II ni es capaz de separar sus cromátidas (Crouse, 1960). El conjunto de estos experimentos llevó a Hellen Crouse (Crouse, 1979) a proponer la existencia de un "elemento controlador" o *"locus CE"* (*"Controlling element"*) que regula la actividad del centrómero en el cromosoma X_m durante la meiosis masculina (Crouse, 1979; Gerbi, 1986). Dicho *locus*, además, es también capaz de controlar la actividad funcional del centrómero de los autosomas. Cabe destacar que no es esencial que el *locus CE* se localice próximo al centrómero del cromosoma receptor para ejercer su control, ya que

en el cromosoma II^X de la translocación T1 (Fig. 6B) el centrómero y los bloques heterocromáticos se localizan en extremos opuestos del cromosoma.

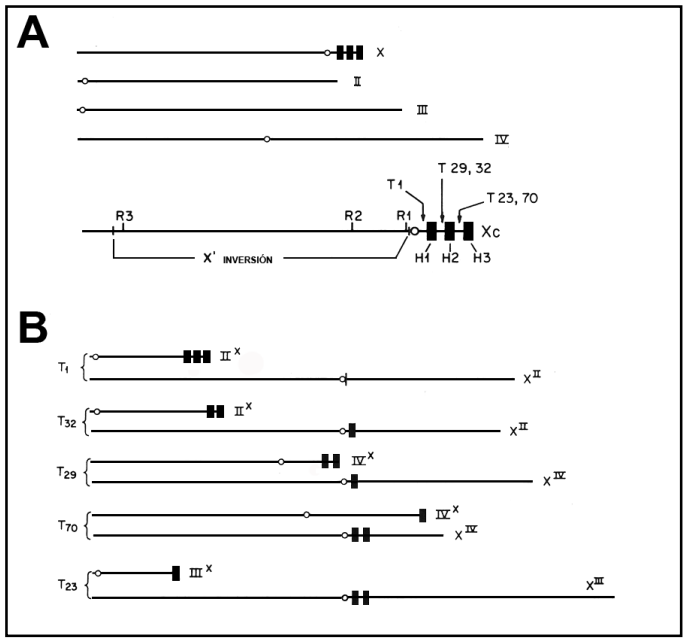

Figura 6. (A) Diagrama del cromosoma X y los tres autosomas de *S. coprophila.* En el cromosoma X se representan los tres bloques heterocromáticos H1, H2 y H3 adyacentes al centrómero. Las flechas indican los puntos de rotura de las translocaciones. **(B) Productos de cinco translocaciones recíprocas** (diagrama según Crouse, 1979).

Por otra parte, la obtención de translocaciones adicionales (Fig. 6B) permitió determinar en cromosomas politénicos la localización del centrómero en una banda específica del cromosoma X (**C** en la Fig. 7) (Crouse, 1977). Así mismo, la heterocromatina adyacente al centrómero del cromosoma X se subdividió en tres bloques o regiones heterocromáticas denominadas H1, H2, y H3 según los puntos de rotura (Fig. 6A) (Crouse, 1979). Estas regiones se extienden desde el centrómero al telómero del cromosoma X (Fig. 7). A pesar de ello, no siempre es posible visualizar citológicamente los tres bloques heterocromáticos de forma lineal. La Figura 6B muestra un esquema de las translocaciones T1, T32, T29, T70, y T23 en las que están implicados un número variable de bloques heterocromáticos. El análisis conjunto de todas estas translocaciones llevó a proponer que el *locus CE* reside en el bloque heterocromático H2 (Crouse, 1977; Crouse, 1979; Abbott y Gerbi, 1981; Gerbi, 2007). No obstante, es importante destacar que no se lograron generar translocaciones en las que se transfiriera únicamente el bloque H2 a los autosomas, por lo que también se puede pensar que la función de H2 requiere de la presencia adicional de H1 o H3. Por

otra parte, cabe destacar que el *locus CE* actúa en *cis* y es capaz de controlar la actividad funcional del centrómero de todos los cromosomas (Crouse, 1979; Gerbi, 2007). Otro dato importante que se infirió del análisis cromosómico de las translocaciones recíprocas entre el cromosoma X y los autosomas en *S. coprophila,* es que el cromosoma X_m que no segrega normalmente en meiosis II es el mismo que en el siguiente ciclo celular es reconocido como paterno y eliminado del soma embrionario (Metz, 1934; Crouse, 1943; Crouse, 1960). A pesar de su importancia funcional, se desconoce tanto el mecanismo de control del *locus CE* como las secuencias específicas de DNA que están involucradas en él. Independientemente de su naturaleza, el *locus CE* es capaz de regular dos procesos diferentes en relación a modificaciones en la segregación del cromosoma X como son: la no disyunción del cromosoma X_m en meiosis II y la eliminación del cromosoma X_p del soma embrionario (Gerbi, 1986; Goday y Esteban, 2001)

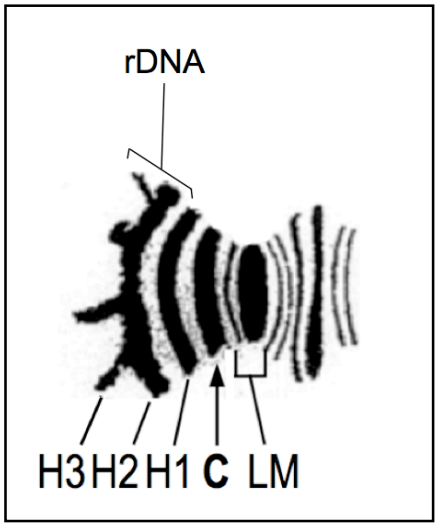

Figura 7. Diagrama del extremo del cromosoma X politénico conteniendo el centrómero en *S. coprophila*. La flecha señala la posición del centrómero **(C**). Se indica la posición de los tres bloques heterocromáticos **H1**, **H2** y **H3**, el DNA ribosómico (**rDNA**), y la región denominada **LM** (*"Land Mark"*; Crouse, 1977).

Mediante hibridación "*in situ*" con sondas de DNA ribosómico sobre cromosomas politénicos, se determinó que los genes ribosomales (rDNA en la Fig. 7) están presentes en los tres bloques heterocromáticos H1, H2 y H3 (Fig. 7) (Crouse *et al.*, 1977). En concreto, aproximadamente un 10% se localiza en H1, 50% en H2, y 40% en H3

(Crouse *et al.*, 1977). En vista de ello, se concluyó que el *locus CE* debe estar embebido entre las repeticiones en tándem del DNA ribosómico (Abbott y Gerbi 1981).

El conjunto de datos existentes en *S. coprophila* indican que la región heterocromática del brazo corto del cromosoma X desempeña un papel fundamental en el control de los procesos básicos de segregación cromosómica. Por ello, el profundizar en el estudio de esta heterocromatina constituye un requisito esencial para conocer su papel tanto en la eliminación de los cromosomas paternos como en la no disyunción del X_m durante la meiosis masculina .

Aspectos relevantes de la heterocromatina

Existen dos tipos de heterocromatina: la constitutiva y la facultativa (Brown, 1966). La heterocromatina constitutiva está presente por igual en todas las células del organismo, localizándose fundamentalmente en el centrómero, en los telómeros y en las regiones organizadoras del nucleolo. Sin embargo, la heterocromatina facultativa varía su estado de condensación en los distintos tipos celulares y en las diferentes etapas del desarrollo (Grewal y Jia, 2007). Un ejemplo de heterocromatina facultativa se encuentra en el corpúsculo de Barr, que se forma como consecuencia de la inactivación de un cromosoma X en las hembras de los mamíferos (Lyon, 1961).

Los cromosomas de *S. coprophila,* comparado con los de *D. melanogaster*, presentan un bajo contenido de heterocromatina constitutiva y por tanto su genoma tiene pocas secuencias de DNA repetido (Abbott *et al.*, 1981).

Aunque la heterocromatina ocupa una fracción considerable del genoma de *D. melanogaster* (del 30-35%), el conocimiento de las secuencias que lo componen y su organización es limitado debido a su naturaleza altamente repetida (Hoskins *et al.*, 2007; Girton y Johansen, 2008). La heterocromatina está compuesta básicamente por secuencias repetidas en tándem denominadas satélites de DNA, por elementos moderadamente repetidos como los transposones y por algunos genes denominados "genes heterocromáticos" (Gatti y Pimpinelli, 1992; Weiler y Wakimoto, 1995; Carvalho *et al.*, 2001; Smith *et al.*, 2007). Los satélites de DNA suelen llegar a formar grandes bloques que están, a su vez, interrumpidos por "islas" de secuencias de mayor complejidad, en su mayoría transposones (Hall *et al.*, 2003). Recientemente se ha demostrado que pequeños RNAs heterocromáticos ("siRNA") dirigen las

modificaciones epigenéticas de las histonas y del DNA (Lippman y Martienssen, 2004; Girton y Johansen, 2008).

Algunas de las funciones claves en las que está implicada la heterocromatina incluyen: la regulación de la segregación cromosómica a través del centrómero y los telómeros (Allshire *et al.*, 1995; Kellum y Alberts, 1995; Peters *et al.*, 2001; Peters *et al.*, 2001), el mantenimiento de la cohesión entre cromátidas hermanas (Wines y Henikoff, 1992; Karpen *et al.*, 1996; Bernard *et al.*, 2001) y el apareamiento de los cromosomas homólogos en meiosis (McKee y Karpen, 1990; Dernburg *et al.*, 1996; Karpen *et al.*, 1996). Actualmente se acepta también que la heterocromatina juega un papel en la organización espacial o "arquitectura" del núcleo interfásico. Así, se ha podido demostrar la asociación de regiones heterocromáticas con sitios específicos de la envuelta nuclear (Croft *et al.*, 1999; Cremer *et al.*, 2001; Cremer y Cremer, 2010).

Los telómeros son estructuras nucleoprotéicas localizadas en los extremos de los cromosomas eucarióticos (Muller, 1938). Los telómeros están implicados en diversas funciones relevantes para el correcto desarrollo del ciclo cromosómico. Entre ellas, cabe destacar, la del mantenimiento de la longitud cromosómica mediante elongaciones periódicas encargadas de contrarrestar la incapacidad de la DNA polimerasa para replicar completamente los cromosomas lineales (Blackburn, 1994; McCord y Broccoli, 2008). Otra de las funciones principales es la de señalizar los extremos naturales del cromosoma, de manera que éstos puedan distinguirse respecto de los extremos generados por las roturas de doble cadena y, por lo tanto, no se activen los mecanismos de reparación, degradación o recombinación (Zhu *et al.*, 1999; McCord y Broccoli, 2008). Además, es importante añadir que los telómeros lideran el movimiento de los cromosomas en la profase meiótica (Rhoades y Vilkomerson, 1942; Östergren y Prakken, 1946; Goday y Pimpinelli, 1989; de Lange, 1992; Gilson *et al.*, 1993; Perez *et al.*, 1997; Manzanero y Puertas, 2003).

Las repeticiones teloméricas están altamente conservadas, desde levaduras (Ogino *et al.*, 1998) hasta protozoos, plantas y vertebrados (Brown *et al.*, 1990; Muller *et al.*, 1991). En general, el telómero de la mayoría de organismos eucariotas está constituido por repeticiones en tándem de una secuencia corta rica en guanina (G) y timina (T) de unas 5-8 pb del tipo (TnGn)n. Si bien la secuencia de la unidad de repetición ha variado a lo largo de la evolución, la riqueza en Gs y su asimetría están conservadas evolutivamente (Meyne *et al.,* 1989). En la mayoría de organismos, el mantenimiento de los extremos de los cromosomas es llevado a cabo por la telomerasa, enzima

ribonucleoprotéico específico de telómeros que añade repeticiones simples por transcripción reversa (Greider, 1996). Este mecanismo está altamente conservado en los eucariotas, desde organismos unicelulares a las plantas y vertebrados (Krupp *et al.*, 2000). Sin embargo, la ausencia de las típicas secuencias repetidas en algunas plantas y en algunos insectos, sugiere que ciertos organismos son capaces de reparar los extremos de los cromosomas utilizando mecanismos independientes de la telomerasa.

En insectos, (TTAGG)n es la secuencia telomérica consenso mas extendida (Okazaki *et al.*, 1993). Sin embargo, el análisis de los telómeros llevado a cabo en insectos del Orden Díptera, y algunas especies de Coleóptera e Hymenóptera, ha revelado que éstos carecen de las típicas repeticiones teloméricas generadas por la telomerasa (Okazaki *et al.*, 1993; Sasaki y Fujiwara, 2000). Se ha observado, además, que en estos organismos han evolucionado diferentes mecanismos encargados del mantenimiento del telómero, que se basan principalmente en la retrotransposición de elementos móviles no-LTR ("*Long Terminal Repeat"*) y en la recombinación desigual de las repeticiones en tándem (Biessmann y Mason, 2003; Pardue y DeBaryshe, 2003). Dentro de los dípteros, los géneros *Drosophila* y *Chironomus* son los que han sido estudiados más rigurosamente.

En *D. melanogaster*, así como en otras especies de *Drosophila*, la formación de los telómeros tiene lugar por retrotransposición de elementos transponibles específicos de telómeros. En éste organismo han sido descritos tres elementos distintos: *HeT-A* (Danilevskaya *et al.*, 1992; Danilevskaya *et al.*, 1994; Mason y Biessman, 1995; Pardue *et al.*, 1996), *TART* (Levis *et al.*, 1993; Sheen y Levis, 1994) y *TAHRE* (Abad *et al.*, 2004; Villasante *et al.*, 2007). Dichos elementos son retrotransposones no-LTR que transponen al final de los telómeros, dejando su extremo 3' más próximo al centrómero y el 5' en el final del cromosoma. Esta orientación en la retrotransposición hace que los telómeros de *D. melanogaster* estén formados por tandems de retrotransposones que dan lugar a repeticiones más largas y complejas que las generadas por la telomerasa (Pardue *et al.*, 1997). Estos elementos mantienen la longitud de los telómeros pero no protegen el final del cromosoma del acortamiento causado por el problema de la replicación incompleta (Mason y Biessman, 1995). Además, estos elementos se caracterizan por tener una región 3' UTR especialmente larga, hecho inusual en los retrotransposones. Tanto *TART* como *TAHRE* tienen dos ORF que codifican para una proteína de unión a ácidos nucleicos y para una retrotranscriptasa respectivamente. *HeT-A*, sin embargo, carece de la ORF (*"Open Reading Frame"*) que codifica la retrotranscriptasa.

Recientemente se ha postulado que *HeT-A* deriva del retrotransposón *TAHRE* (con el que tiene gran homología) mediante la pérdida de su ORF II (Abad *et al.*, 2004). Así, el elemento *HeT-A*, carente de retrotranscriptasa, se transpone gracias a una retrotranscriptasa suministrada en *trans*, probablemente la de *TAHRE*.

Los telómeros de *Chironomus* son un modelo interesante, ya que hasta ahora no se han encontrado en este género las típicas repeticiones teloméricas, ni tampoco retrotransposones. En su lugar se han encontrado familias de secuencias complejas repetidas en tándem (Lopez *et al.*, 1999). En los Chironómidos siete de los ocho cromosomas comparten secuencias teloméricas, y en el único cromosoma acrocéntrico, el IV, las secuencias teloméricas han sido sustituidas por secuencias centroméricas (Rovira *et al.*, 1993; Rosen *et al.*, 2002). Los tamaños de dichas secuencias van de 176-360 pb, siendo el tamaño y el tipo de secuencias especie específico (Lopez *et al.*, 1999). Sin embargo, el descubrimiento de una transcriptasa reversa asociada a los telómeros hizo pensar en la implicación de ésta en el mantenimiento de los telómeros en *Chironomus* (Diez *et al.*, 2006). Cabe destacar que en otros dípteros (*Anopheles)* y también en plantas (*Allium* y *Aloe*) han sido descritas secuencias teloméricas complejas semejantes a las encontradas en este organismo (Biessmann *et al.*, 1996; Biessmann *et al.*, 2000; Sykorova, Lim *et al.*, 2003).

En los Ciáridos los datos existentes sobre los telómeros provienen de estudios realizados en la especie *Rhynchosciara americana*, donde en las regiones terminales no centroméricas han sido identificadas diferentes tipos de secuencias en tándem poco comunes, así como también proteínas relacionadas con la regulación de la transcripción reversa. Basado en estos dos hechos se ha propuesto que en esta especie el mantenimiento de los telómeros es llevado a cabo también mediante transcripción reversa (Gorab, 2003).

Otro elemento clave en el control del comportamiento cromosómico es el centrómero. Se trata de la región del cromosoma especializada en dirigir la segregación de las cromátidas hermanas a las células hijas durante la mitosis. El centrómero se encuentra en la constricción primaria en los cromosomas mitóticos de los eucariotas superiores. Es en el centrómero donde se ensambla el cinetocoro, estructura proteica compleja que interacciona con los microtúbulos durante la segregación cromosómica. En los organismos eucariotas en general, desde levaduras hasta humanos, el centrómero está situado en regiones cromosómicas heterocromáticas (Grewal y Jia, 2007). La heterocromatina que rodea a los centrómeros, conocida como heterocromatina

pericentromérica, desempeña un papel esencial en la segregación de los cromosomas, ya que defectos en la formación de ésta tienen como consecuencia una segregación aberrante de los cromosomas (Allshire *et al.*, 1995; Kellum y Alberts, 1995; Peters *et al.*, 2001). Por otro lado, diversas observaciones sugieren que la heterocromatina pericentromérica también juega un papel importante en el mantenimiento de la cohesión entre cromátidas hermanas (Wines y Henikoff, 1992; Karpen *et al.*, 1996). Aunque se conoce muy poco sobre el mecanismo molecular responsable de este fenómeno, en estudios llevados a cabo en *S. pombe* se ha demostrado que existe interacción entre algunas proteínas heterocromáticas y las proteínas del complejo de cohesión o cohesinas (Bernard *et al.*, 2001; Nonaka *et al.*, 2002).

En eucariotas superiores el DNA centromérico se caracteriza por su alto contenido en secuencias repetidas en tándem o satélites de DNA. En particular, en humanos se ha visto que todos sus centrómeros tienen bloques del denominado satélite alfoide (de hasta 7 Mb) con unidades de 171 bp (Maio, 1971; Mitchel *et al.*, 1985; Choo *et al.*, 1991).

Aunque la maquinaria proteica responsable de la correcta segregación cromosómica se encuentra conservada, la naturaleza repetida de las secuencias centroméricas hace que éstas evolucionen rápidamente y que por lo tanto no se encuentren conservadas. Por otra parte, los estudios llevados a cabo sobre neocentrómeros han mostrado que en ocasiones los centrómeros pueden formarse en regiones que carecen de las secuencias centroméricas conocidas, y que sin embargo son capaces de formar un cinetocoro. Estos datos sugieren que la formación de un centrómero en un lugar determinado requiere mecanismos epigenéticos específicos (Karpen y Allshire, 1997). Además, Wong y colaboradores han sugerido que los transcritos de los satélites centroméricos podrían facilitar el ensamblaje de la cromatina centromérica (Wong *et al.*, 2007).

Los datos que se conocen sobre los centrómeros en dípteros corresponden a *D. melanogaster*, donde diferentes estudios citogenéticos permitieron localizar los satélites de DNA presentes en su genoma. De los resultados de estos estudios se concluyó que la mayoría de los satélites se encuentran en varias localizaciones no centroméricas, sin que se encontrara ningún satélite común a todos los centrómeros (Pimpinelli *et al.*, 1994; Carmena y Gonzalez, 1995). El estudio realizado sobre el centrómero del minicromosoma Dp1187, derivado del cromosoma X, delimitó la función centromérica a 420 kb (Murphy y Karpen, 1995; Sun *et al.*, 1997). En esa región se observó la presencia de dos satélites de DNA no específicos de centrómero con unidades de

repetición $(AAGAG)_n$ y $(AATAT)_n$ respectivamente. Sin embargo, los estudios llevados a cabo en otros cromosomas han llevado a encontrar satélites centroméricos específicos: en el cromosoma Y se ha descrito el satélite 18HT (Mendez-Lago *et al.*, 2009), mientras que en el cromosoma 3 se ha descrito el satélite dodeca (Abad *et al.,* 1992). De esta manera, *D. melanogaster* parece tener secuencias centroméricas diferentes para cada cromosoma. Del análisis comparativo del conjunto de datos de los centrómeros de *D. melanogaster*, vertebrados y plantas se concluye que existe una organización centromérica común consistente en islas de transposones embebidas en grandes regiones de repeticiones simples o complejas (Sun *et al.*, 2003; Nagaki *et al.*, 2004), y esta organización es notablemente similar a la existente en las regiones subteloméricas de todos los cromosomas eucarióticos, consistentes en mosaicos de repeticiones y retrotransposones (Pryde *et al.*, 1997).

Como ya se ha mencionado anteriormente, se desconoce la organización molecular de las regiones heterocromáticas de los cromosomas de *S. coprophila,* incluyendo el *locus CE*. Una posible estrategia para comenzar el análisis molecular de la heterocromatina de *S. coprophila* podría ser la microdisección de regiones heterocromáticas de sus cromosomas politénicos, pues este tipo de abordaje experimental ha sido realizado con éxito en diferentes insectos incluyendo *Drosophila* (Scalenghe *et al.*, 1981; Pirrotta *et al.*, 1983; Moshkin *et al.*, 2002).

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Objetivos

OBJETIVOS

El objetivo del presente trabajo es el estudio celular y molecular de la heterocromatina del díptero *Sciara.* En particular el trabajo se centra en:

1) Caracterización de las secuencias de DNA de la heterocromatina centromérica del cromosoma X de *Sciara coprophila.*

- Microdisección de la región heterocromática del extremo centromérico del cromosoma X.
- Obtención de las librerías correspondientes a la región micro-diseccionada.
- Secuenciación y análisis de los clones.
- Localización de las secuencias aisladas mediante hibridación *"in situ"* sobre cromosomas politénicos, mitóticos y meióticos.

2) Análisis de las modificaciones epigenéticas de la cromatina, en concreto de la fosforilación de la histona H3 en la región centromérica del cromosoma X en la meiosis masculina en *Sciara*.

- Determinar los patrones de fosforilación de la histona H3 durante las distintas fases de la meiosis en *S. ocellaris* y *S. coprophila* usando anticuerpos que reconocen la fosforilación de la histona H3 en las serinas S10 y S28, y en las treoninas T3 y T11.

3) Caracterización de secuencias teloméricas de *Sciara coprophila.*

- Microdisección de la región telomérica 1A del cromosoma IV.
- Obtención de la librería correspondiente.
- Secuenciación y análisis de los clones.
- Localización de las secuencias aisladas mediante hibridación *"in situ"* sobre cromosomas politénicos.

Resultados

1

"Caracterización citológica y molecular de secuencias repetidas de DNA de la heterocromatina centromérica de *S. coprophila*"

Resumen

Sciara coprophila (Diptera, Nematocera) constituye un modelo clásico para el estudio de comportamientos cromosómicos inusuales como son la eliminación del complemento cromosómico paterno completo y la no-disyunción del cromosoma X materno durante la meiosis masculina. Se desconoce la organización molecular de la heterocromatina de *S. coprophila*, a excepción del rDNA, que se encuentra localizado en la heterocromatina pericentromérica del cromosoma X. Por esta razón, la caracterización molecular de las regiones centroméricas es un paso necesario para el establecimiento de *S. coprophila* como sistema modelo para el estudio de los mecanismos fundamentales de la segregación cromosómica. Para llevar a cabo este estudio, se microdiseccionaron y microclonaron secciones de la heterocromatina centromérica del cromosoma X. La microdisección se realizó en cromosomas politénicos de las glándulas salivales. En el presente trabajo se ha llevado a cabo la identificación y caracterización de dos secuencias repetidas en tándem, un elemento transponible (*"RTE")* pericentromérico, y un satélite centromérico rico en AT. Estas secuencias podrán ser utilizadas como herramientas para clonar la heterocromatina centromérica de S. coprophila utilizando genotecas genómicas con insertos de gran tamaño.

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RESEARCH ARTICLE

Molecular and cytological characterization of repetitive DNA sequences from the centromeric heterochromatin of *Sciara coprophila*

M. Carmen Escribá · Patricia G. Greciano · María Méndez-Lago · Beatriz de Pablos · Vladimir A. Trifonov · Malcolm A. Ferguson-Smith · Clara Goday · Alfredo Villasante

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Abstract *Sciara coprophila* (Diptera, Nematocera) constitutes a classic model to analyze unusual chromosome behavior such as the somatic elimination of paternal X chromosomes, the elimination of the whole paternal, plus non-disjunction of the maternal X chromosome at male meiosis. The molecular organization of the heterochromatin in *S. coprophila* is mostly unknown except for the ribosomal DNA located in the X chromosome pericentromeric heterochromatin. The characterization of the centromeric regions, thus, is an essential and required step for the establishment of *S. coprophila* as a model system to study fundamental mechanisms of chromosome segregation. To accomplish such a study, heterochromatic sections of the X chromosome centromeric region from salivary glands polytene chromosomes were microdissected and microcloned. Here, we report the identification and characterization of two tandem repeated DNA sequences from the pericentromeric region of the X chromosome, a pericentromeric RTE element and an AT-rich centromeric satellite. These sequences will be important tools for the cloning of *S. coprophila* centromeric heterochromatin using libraries of large genomic clones.

Introduction

The lower dipteran fly *Sciara coprophila* is an excellent model organism to study chromosome behavior due to its unique biology. Among the unusual biological processes

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M. C. Escribá · M. Méndez-Lago · B. de Pablos · A. Villasante (✉)
Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM),
Universidad Autónoma de Madrid,
Nicolás Cabrera 1,
28049 Madrid, Spain
e-mail: avillasante@cbm.uam.es

M. C. Escribá · P. G. Greciano · C. Goday (✉)
Departamento de Proliferación Celular y Desarrollo,
Centro de Investigaciones Biológicas,
CSIC, Ramiro de Maeztu 9,
28040 Madrid, Spain
e-mail: claragoday@cib.csic.es

V. A. Trifonov · M. A. Ferguson-Smith
Department of Veterinary Medicine,
Cambridge Resource Centre for Comparative Genomics,
Cambridge, UK

V. A. Trifonov
Molecular and Cellular Biology Department,
Institute of Chemical Biology and Fundamental Medicine,
SB RAS,
630090 Novosibirsk, Russia

Present Address:
P. G. Greciano
Department of Surgery, Committee on Cell Physiology, and
Committee on Molecular Pathogenesis and Molecular Medicine,
The University of Chicago,
Chicago, IL 60637, USA

Present Address:
M. Méndez-Lago
Genome Sciences Center,
British Columbia Cancer Research Center,
Vancouver, BC, Canada

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are the selective elimination of one or both paternal X chromosomes from the nuclei of the developing embryo, the elimination of the paternal chromosomes at male meiosis I, and the non-disjunction of the maternal X chromosome at male meiosis II (Metz 1925, 1926, 1938; reviewed in Gerbi 1986; Goday and Esteban 2001).

The somatic nuclei of *S. coprophila* contain three pairs of autosomes (chromosomes II, III, and IV) and one or two X chromosomes depending on the sex (males are X0 and females XX). Chromosomes II, III, and X are acrocentric, while chromosome IV, the largest one, is metacentric. A distinctive feature of *S. coprophila* is the existence of additional metacentric chromosomes, mostly heterochromatic, denominated "L" chromosomes. L chromosomes, generally two or three per nucleus, are restricted to the germline, since they are all discarded during early cleavages by a process similar to that of paternal X chromosomes elimination (Rieffel and Crouse 1966).

The approximate positions of the centromeres were identified on the four polytene chromosomes by means of reciprocal translocations (Crouse 1943, 1977). Similarly, the X heterochromatin containing the ribosomal DNA (rDNA) (Gerbi 1971) was subdivided by translocations into three blocks (H1, H2, and H3) extending from the X centromere to the telomere (Crouse et al. 1977). These blocks constitute the short heterochromatic arm of the X and correspond to the three structural elements immediately adjacent to the centromere. However, it is difficult to see a linear arrangement of the elements due to their variable cytological appearance. Approximately, 10% of the rDNA are located in H1, 50% in H2, and 40% in H3 (Crouse et al. 1977). Interestingly, the middle block (H2) contains also the *cis*-acting locus (controlling element) responsible for both the nondisjunction of the maternal X chromosome in male meiosis and the X chromosome somatic elimination in early embryogenesis (Crouse 1979; Gerbi 2007).

Little is known about the molecular organization of the heterochromatin of *S. coprophila* except for some sequence information about the rDNA (Gerbi and Crouse 1976; Renkawitz et al. 1979; Jordan et al. 1980; Renkawitz-Pohl et al. 1981; Ware et al. 1985; Kerrebrock et al. 1989; Burke et al. 1993). Satellite DNA present in the centromeric heterochromatin has been identified (Abbott et al. 1981; Abbott and Gerbi 1981) but not sequenced. However, characterization of the centromeric regions is an inevitable and essential step required for the establishment of *S. coprophila* as a model system to study fundamental mechanisms of chromosome segregation. Therefore, taking into account that the *S. coprophila* heterochromatin does not form a chromocenter in polytene nuclei and that this allowed the construction of a cytogenetic map of the X heterochromatin, we decided to microdissect the centromeric region of the X polytene chromosome as an initial approach to isolate centromeric sequences. Here, we present the initial characterization of four repetitive DNA sequences, two of them with a pancentromeric distribution. These sequences will be instrumental for the cloning of *S. coprophila* centromeric heterochromatin using libraries of large genomic clones.

Materials and methods

Fly culture

S. coprophila flies (stock 6980 with Wavy and swollen markers) were raised at 20°C as described elsewhere Rieffel and Crouse (1966).

Chromosome microdissection and microcloning

S. coprophila polytene chromosomes preparations were obtained by squashing salivary glands from fourth-instar larvae in 45% acetic acid. Glass needle-based microdissection of specific X chromosome heterochromatic regions was carried out using an inverted microscope (Axiovert 135, Zeiss) with the help of a micromanipulator. The microdissected chromosome fragments were transferred by inserting the tip of the needle into the micropipette containing the collection solution: 30% glycerol, 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 0.1% SDS, 0.1%Triton X-100, 1 mM EDTA, and 1.4 mg/ml proteinase K. The micropipette containing the chromosome fragments was incubated in a water bath at 60°C for 2 h. The dissected chromosomal DNA was amplified by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) as described (Telenius et al. 1992). The amplified DNAs were cloned into the TOPO TA-cloning vector (Invitrogen).

Chromosome preparations and fixation

Salivary glands from fourth-instar larvae and prepupae testes were dissected in 15 mM Tris–HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, and 0.1% Triton X-100 solution and immediately transferred to a drop of 45% acetic acid on a siliconized coverslip and squashed. Neuroblasts from third-instar larvae were dissected in the same buffer, and prior to squashing in a drop of 45% acetic acid, they were incubated in 17 mM sodium citrate, pH 7.0 for 10 min. After freezing in liquid nitrogen and removing the coverslips, all slides were post-fixed in absolute ethanol for 10 min; they were then air-dried and kept at room temperature.

Fluorescence in situ hybridization (FISH)

Air-dried preparations were pre-treated in 2× SSC at 65°C for 30 min (1× SSC is 150 mM NaCl, 15 mM

sodium citrate), dehydrated in 70% and 95% ethanol and air-dried. Chromosomal DNA was denatured in 0.07 N NaOH for 2.5 min, washed three times in 2× SSC, dehydrated again, and dried in air. The hybridization mixture was 50% (*v*/*v*) formamide, 4× SCC, 10% (*w*/*v*) dextran sulfate, and 0.4% SDS. Hybridization was performed overnight at 37°C. Slides were then washed four times for 5 min in 2× SSC at room temperature, followed by three washes in 0.2× SSC at 40°C. Probes were labeled by nick translation with either digoxigenin 11-dUTP or biotin 16-dUTP (Roche Diagnostic GmbH). Digoxigenin-labeled probes and biotinylated probes were detected with anti-digoxigenin-fluorescein, Fab fragments (Roche) and Texas red-streptavidin (Molecular Probes), respectively. Chromosomes were counterstained with 4′,6-diamino-2-phenylindole. Observations were made under epifluorescence optics with a Zeiss Axiophot microscope equipped with a cooled charge-coupled device camera. The fluorescent signals were recorded separately as grey-scale digital images and then pseudo-colored and merged using Adobe Photoshop software.

Preparation and analysis of DNA

S. coprophila genomic DNA was obtained from adult flies as described (Losada et al. 1997). Plasmid DNA and phage DNA were prepared according to Sambrook et al. (1989). Restriction enzyme digestions were performed following the suppliers' instructions. For restriction enzyme analysis, both conventional agarose gel electrophoresis and pulsed-field gel electrophoresis were used. The DNA from agarose gels was transferred to Hybond N^+ nylon filters (Amersham) in 0.4 M NaOH and hybridized to ^{32}P-labeled probes. The *S. coprophila* genomic library made by Serna et al. (2004) was screened by colony hybridization with probe F4, the PCR-amplified insert of clone X.01F4. DNA probes were labeled by random priming with [^{32}P] dATP using Megaprime DNA labeling system (Amersham). Colony hybridization and

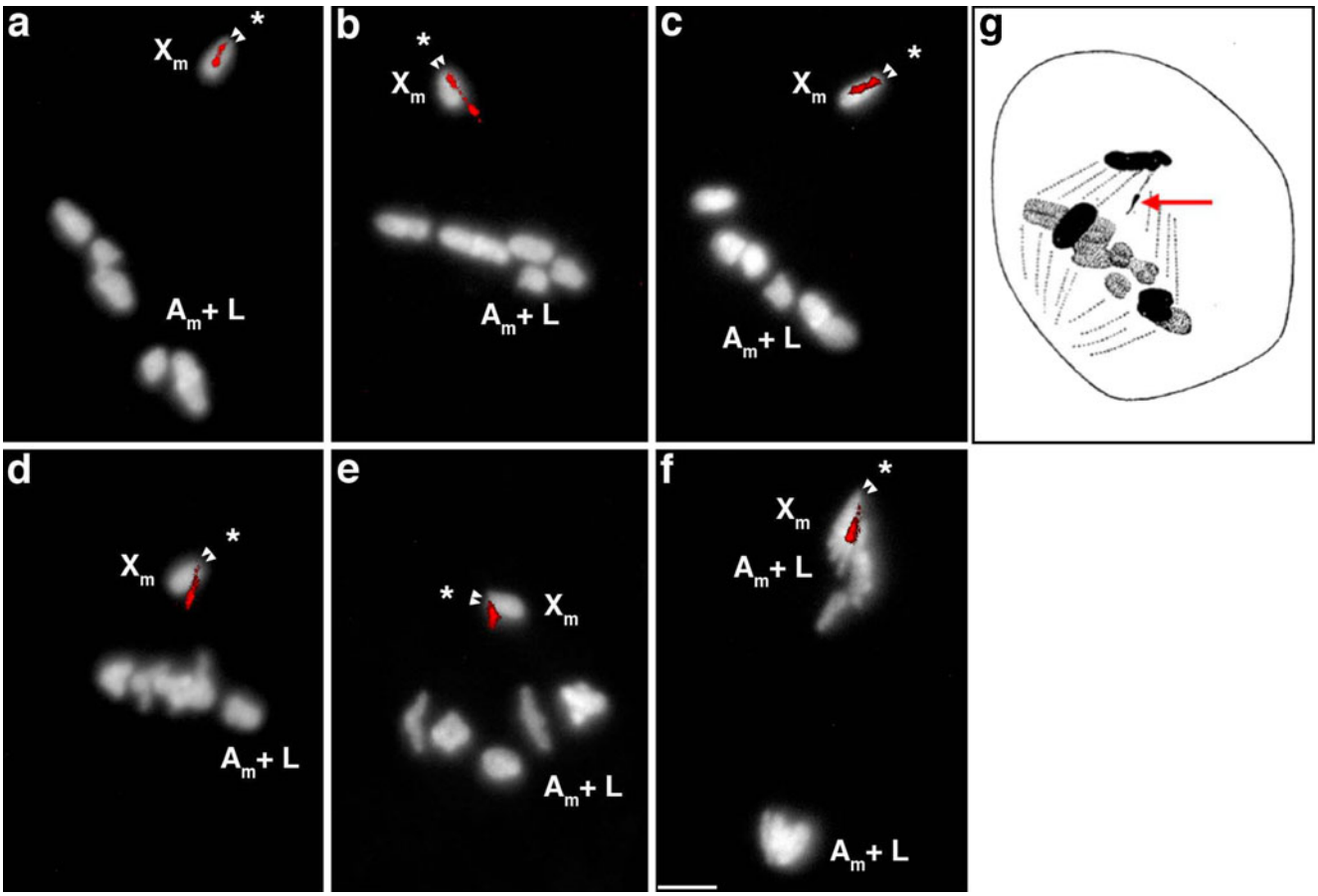


Fig. 1 rDNA in situ hybridization in *S. coprophila* spermatocytes at the second meiotic division. Metaphase II (**a**–**c**), early anaphase II (**d**, **e**), and late anaphase II (**f**) showing the rDNA locus (*red*) at the X_m chromosome; *double arrowheads* indicate the position of the X_m centromeres facing the polar complex. In all images, a significant stretching of the rDNA locus towards the equatorial region of the spermatocytes is observed. **g** A classic diagram of *S. coprophila* male meiosis II where the distortion of the X_m chromosome chromatin at the centromeric end is evident (*arrow*; modified from Metz 1926). X_m maternal X chromosome, A_m maternal autosomal set, *L* germline-limited chromosomes; *asterisks* indicate the deduced position of the polar complex generated at the first meiotic division. *Scale bar* 10 μm

Southern blot hybridization analysis were performed overnight at 65°C in 0.5 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA. Filters were washed with 0.2× SSC; 0.1% SDS at 65°C and exposed to X-ray films. PCR amplification was used to obtain the centromeric DNA repeats from *S. coprophila* genomic DNA. The oligonucleotide primers used in the amplification reaction were: 5′-TATTTTAGCTGGTTG TAAATG-3′ and 5′-CTTTTTTATTTCTGAAATTTTC-3′. The amplification conditions were: an initial denaturation step at 94°C for 5 min, 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C followed by a final extension at 72°C for 10 min. PCR fragments were cloned into the TOPO TA-cloning vector (Invitrogen).

DNA sequencing and sequence analysis

DNA sequencing was carried out using the standard dye terminator chemistry and reactions were analyzed in an ABI Prism 3730 Sequencer (Applied Biosystems, Foster City, CA, USA) at the sequencing facility of the Unidad de Genómica "Antonia Martín Gallardo", PCM-UAM. Primer walking was used to sequence the insert of phage 8.4.3.2. Homology searches were performed by BLAST at web servers of the National Center for Biotechnology Information, Bethesda, MD, USA. Sequence alignments were done with CLUSTALX (Thompson et al. 1997), followed by manual adjustments of gaps. The phylogenetic tree was generated with the MEGA 2.1 program (Kumar et al. 2001) using a neighbor-joining method with bootstrap statistics (1,000 iterations).

Results and discussion

The centromeric regions of two male polytene X chromosomes were microdissected and the chromosomal

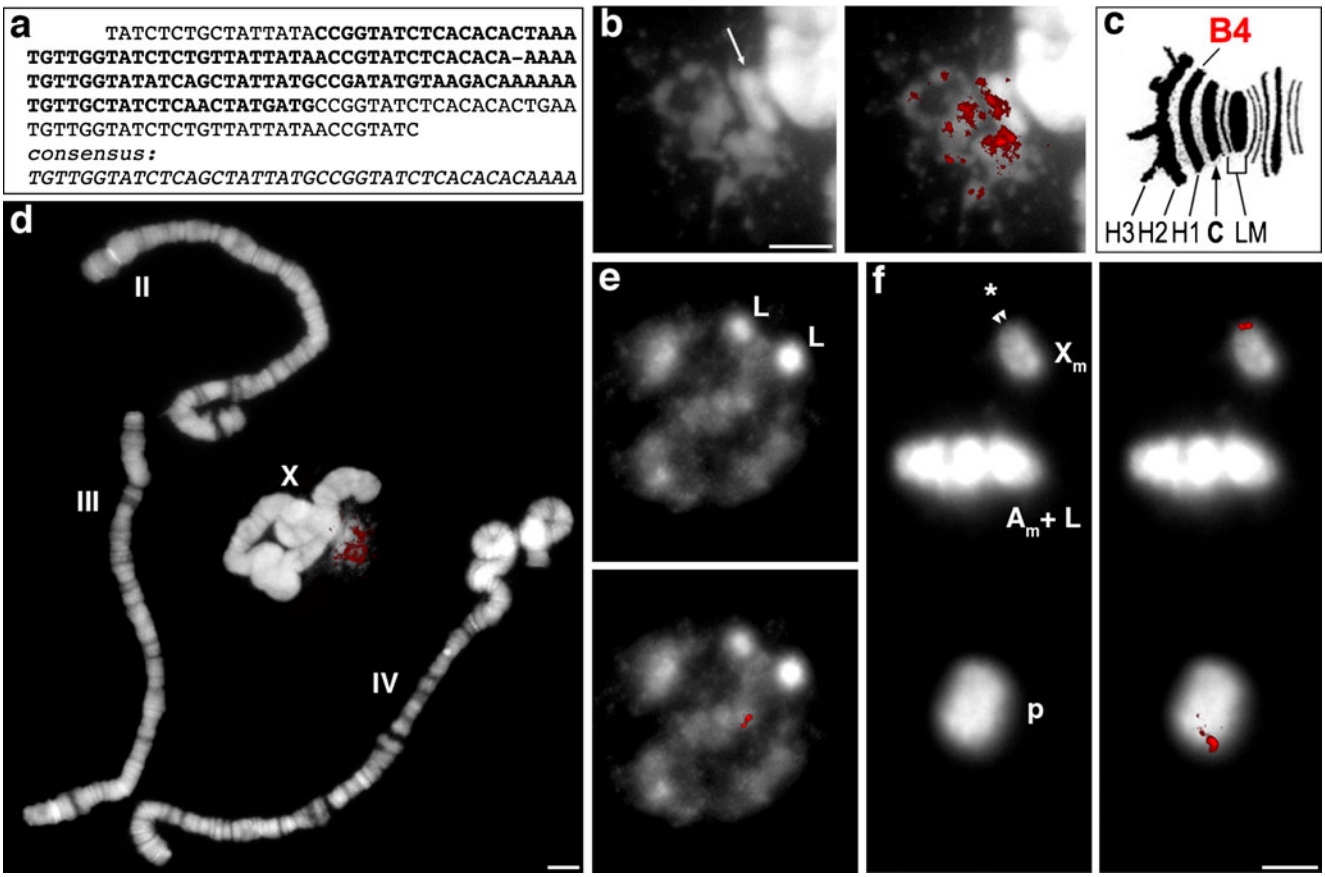


Fig. 2 B4 in situ hybridization in *S. coprophila* polytene chromosomes (**b**–**d**), a germline premeiotic nucleus (**e**), and a spermatocyte at meiosis II (**f**). **a** Sequence of B4 (the X.01B4 insert) showing the 42 bp tandem repeats; the consensus sequence of the 42 bp repeat is indicated in *italics*; the 125 bp higher-order repeat unit is indicated in *bold*. **b** A detailed view of the X chromosome centromeric end showing the location of B4 with respect to the centromere (*arrow*). **c** Schematic diagram of the X chromosome centromeric end (Gabrusewycz-Garcia 1964; Crouse et al. 1977) showing the location of B4 (*red*) at H1. **d** B4 localizes exclusively at the X chromosome and no signals are present in the rest of the chromosomes (II, III, and IV). **e** Premeiotic nucleus showing intranuclear B4 signals corresponding to the X chromosomes. **f** At metaphase II, two double B4 signals can be observed at the X_m chromosome centromere (*arrowheads*). The B4 signal in p corresponds to the X chromosome in the eliminated paternal set as a bud in the first meiotic division. X_m maternal X chromosome, A_m maternal autosomal set, *p* paternal chromosomes, *L* germline-limited chromosomes; *asterisk* indicates the deduced position of the polar complex. *Scale bar* 10 μm

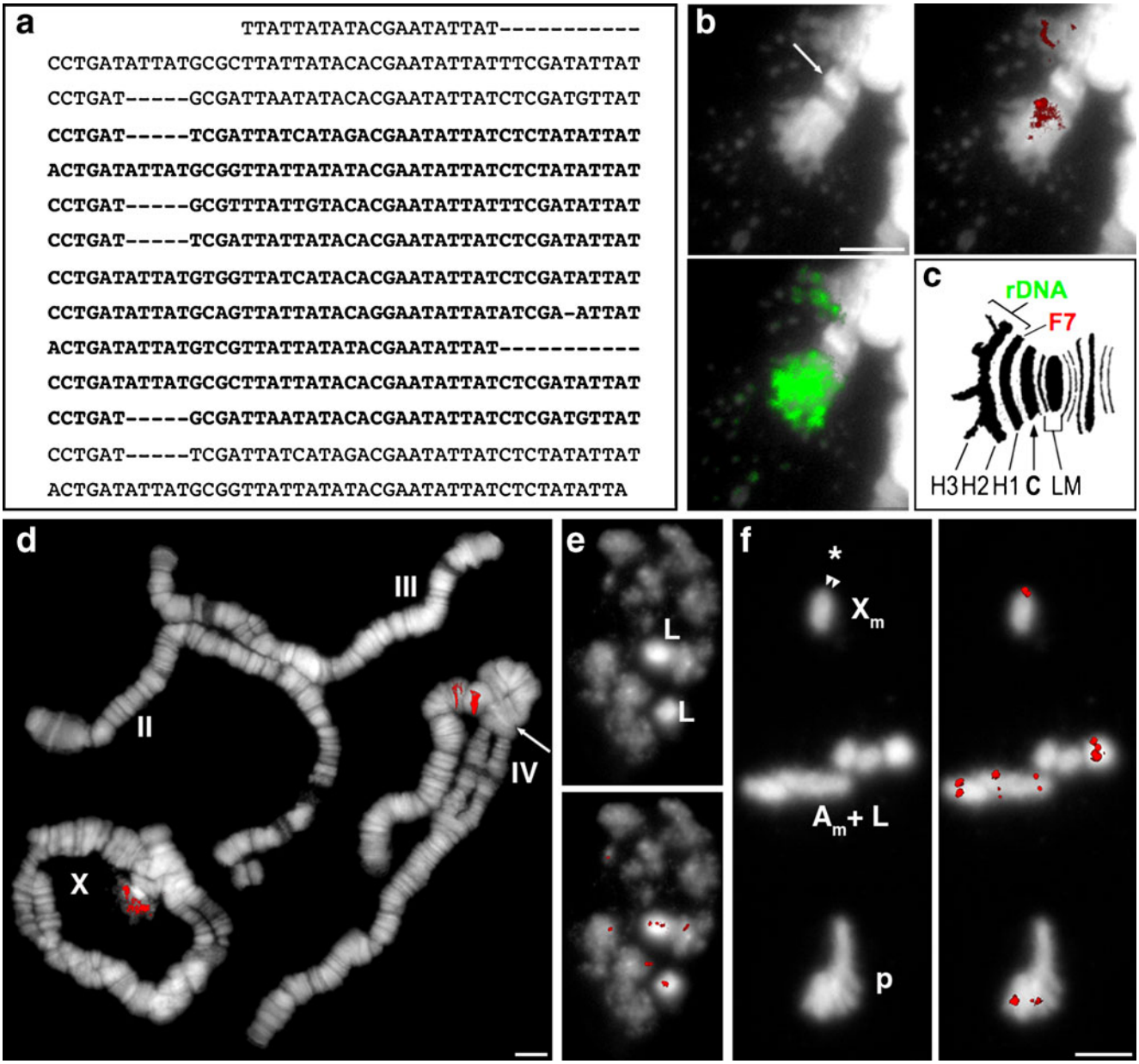


Fig. 3 F7 in situ hybridization in *S. coprophila* polytene chromosomes (**b**–**d**), a germline premeiotic nucleus (**e**), and a spermatocyte at meiosis II (**f**). **a** Sequence of F7 (the X.01F7 insert) showing the 382 bp tandem repeats (in *bold*); the sequence is aligned to make evident the imperfect 46 bp subrepeats. **b** A detailed view of the X chromosome centromeric end showing the location of F7 (*red*) and rDNA (*green*) with respect to the centromere (*arrow*); note the splitting of the chromosome end after the squashing procedure. **c** Schematic diagram of the X chromosome centromeric end (Gabrusewycz-Garcia 1964; Crouse et al. 1977) showing the location of F7 (*red*) at H1. **d** In addition to the X chromosome, F7 is also present at two non-centromeric bands on the IV chromosome (*arrow* point to the centromere). **e** Premeiotic nucleus showing intranuclear F7 signals corresponding to the X, IV, and L chromosomes. **f** At metaphase II, two discrete F7 signals can be discerned at the X_m chromosome; *double arrowheads* denote the position of the centromere facing the polar complex. The F7 signals in the chromosomes at the metaphase plate correspond to the IV and L chromosomes. The F7 signals in p correspond to the X and IV chromosomes of the paternal set eliminated as a bud in meiosis I. X_m maternal X chromosome, A_m maternal autosomal set, *p* paternal chromosomes, *L* germline-limited chromosomes; *asterisk* indicates the deduced position of the polar complex. *Scale bar* 10 μm

DNA was amplified by DOP-PCR. Then, the amplified DNA fragments were used to construct two plasmid libraries. A total of 461 clones were generated and 192 were sequenced. Since the DOP-PCR technique is very sensitive to contamination, we used BLAST analysis to discard some of the clones derived from non-specific DNA amplification. Finally, the screening of 66 clones by FISH on polytene chromosomes has allowed the identification of four clones (X.01B4, X.01F4, X.01F7, and X0.1G2) that hybridized at the centromeric or

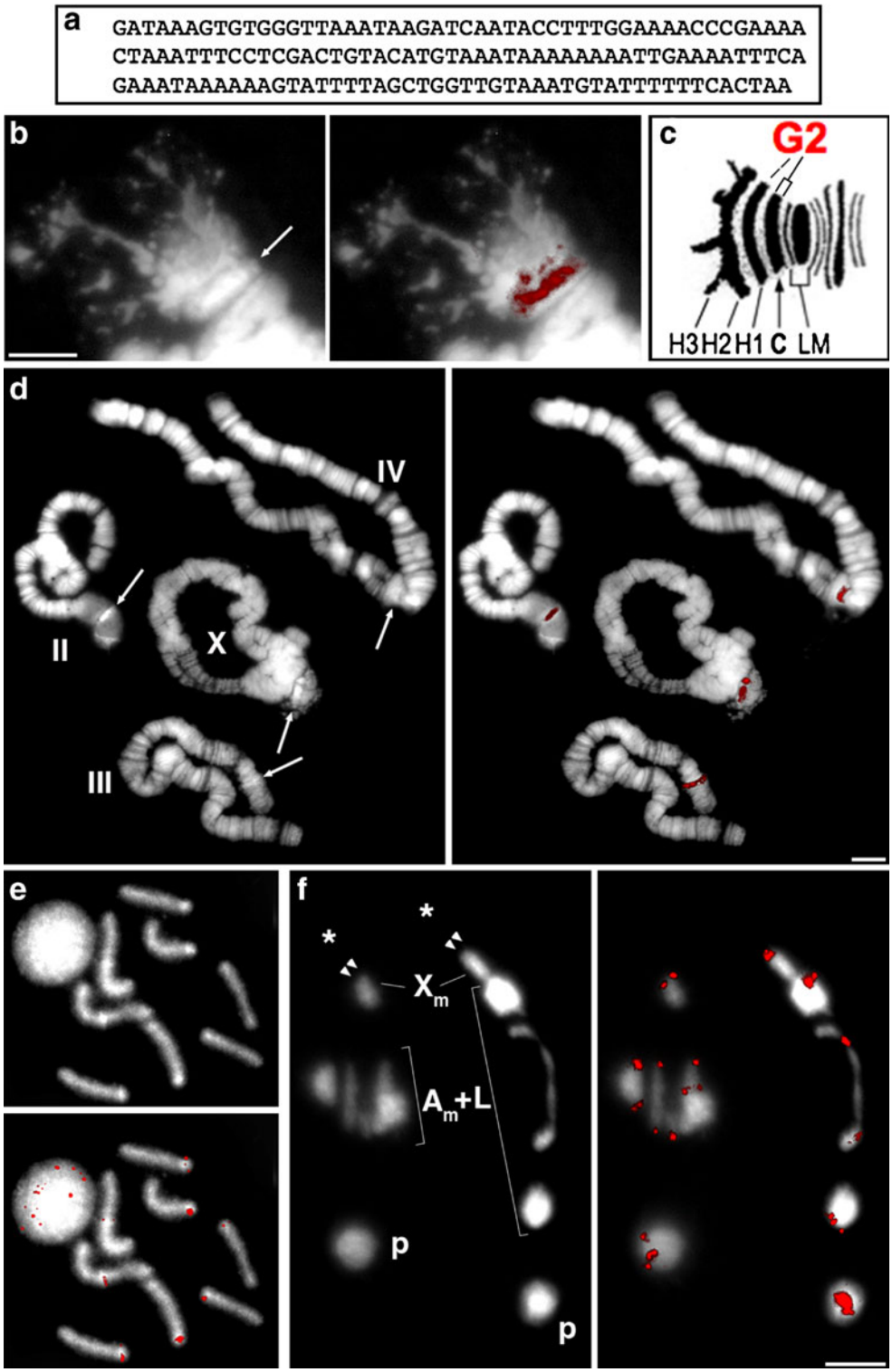


Fig. 4 G2 in situ hybridization in *S. coprophila* polytene chromosomes (**b**–**d**), neuroblasts mitotic chromosomes (**e**), and two spermatocytes at meiosis II (**f**). **a** Sequence of G2 (the X.01G2 insert). **b** A detailed view of the X chromosome centromeric end showing G2 predominantly located at the centromere (*arrow*). **c** Schematic diagram of the X chromosome centromeric end (Gabrusewycz-Garcia 1964; Crouse et al. 1977) showing the location of G2 (*red*) at the centromere (*C*) and, at a minor extent, at H1. **d** G2 signals are also present at the centromeric region of all chromosomes (*arrows*). **e** Neuroblast mitotic chromosomes showing G2 signals in all centromeres. **f** Two spermatocytes at early and late anaphase II (left and right, respectively) where G2 signals are present at the centromeres of all chromosomes; *double arrowheads* denote the position of the centromere facing the polar complex. The G2 signals in p correspond to the centromeres of the paternal set eliminated as a bud in meiosis I. X_m maternal X chromosome, A_m maternal autosomal set, *p* paternal chromosomes, *L* germline-limited chromosomes; *asterisk* indicates the deduced position of the polar complex. *Scale bar* 10 μm

pericentromeric regions of the X. As expected, rDNA clones were also recovered.

Despite the fact that FISH on salivary gland polytene chromosomes allows high-resolution mapping of heterochromatin, the presence of nucleolar material at the centromeric end of the X chromosome hinders that process. As already mentioned, the alignment of the three structural elements H1, H2, and H3 (see diagram in Fig. 2c) is rarely observed and, instead, there is an aggregate of threads and granules. The inconsistent morphology of these three bands prevents a clear assignment of target sites close to the ribosomal genes. Fortunately, the total extension of the

rDNA during the second meiotic division (Fig. 1a–f) allows unambiguous locations of probes in relation to the rDNA locus. The distortion produced in the short arms of the maternal X chromosome by a spindle fiber extending toward the opposite pole (Fig. 1g) has already been described by Metz (1938).

Two tandem repeated DNA sequences in the pericentromeric region of the X chromosome

The insert of clone X.01B4 (B4) is composed of tandem repeats of 42 bp that seem to be organized into a higher-order repeat unit of 125 bp (Fig. 2a). FISH analyses of polytene and meiotic chromosomes revealed that these repeats appear to be primarily in the pericentromeric region H1 (Fig. 2b–f).

The insert of X.01F7 (F7) is composed of tandem repeats of 382 bp, although it is possible to recognize imperfect subrepeats of 46 bp (Fig. 3a). In this case, however, FISH on polytene chromosomes showed that F7 appears to be in block H1, and in two non-centromeric regions of chromosome IV (Fig. 3b–d). In addition, FISH analyses of premeiotic and meiotic chromosomes showed that F7 also hybridizes with the L chromosomes (Fig. 3e, f). This is the first identification of some sequences on the *S. coprophila* L chromosomes.

An AT-rich satellite DNA is present in all centromeres of *S. coprophila*

The nucleotide sequence of the 143 bp insert of X.01G2 (G2) does not show internal repetition (Fig. 4a), and FISH on polytene chromosomes showed that G2 appears to be primarily on all the chromosomes at the centromeric regions mapped by Crouse (1943, 1977) (Fig. 4d). In the X

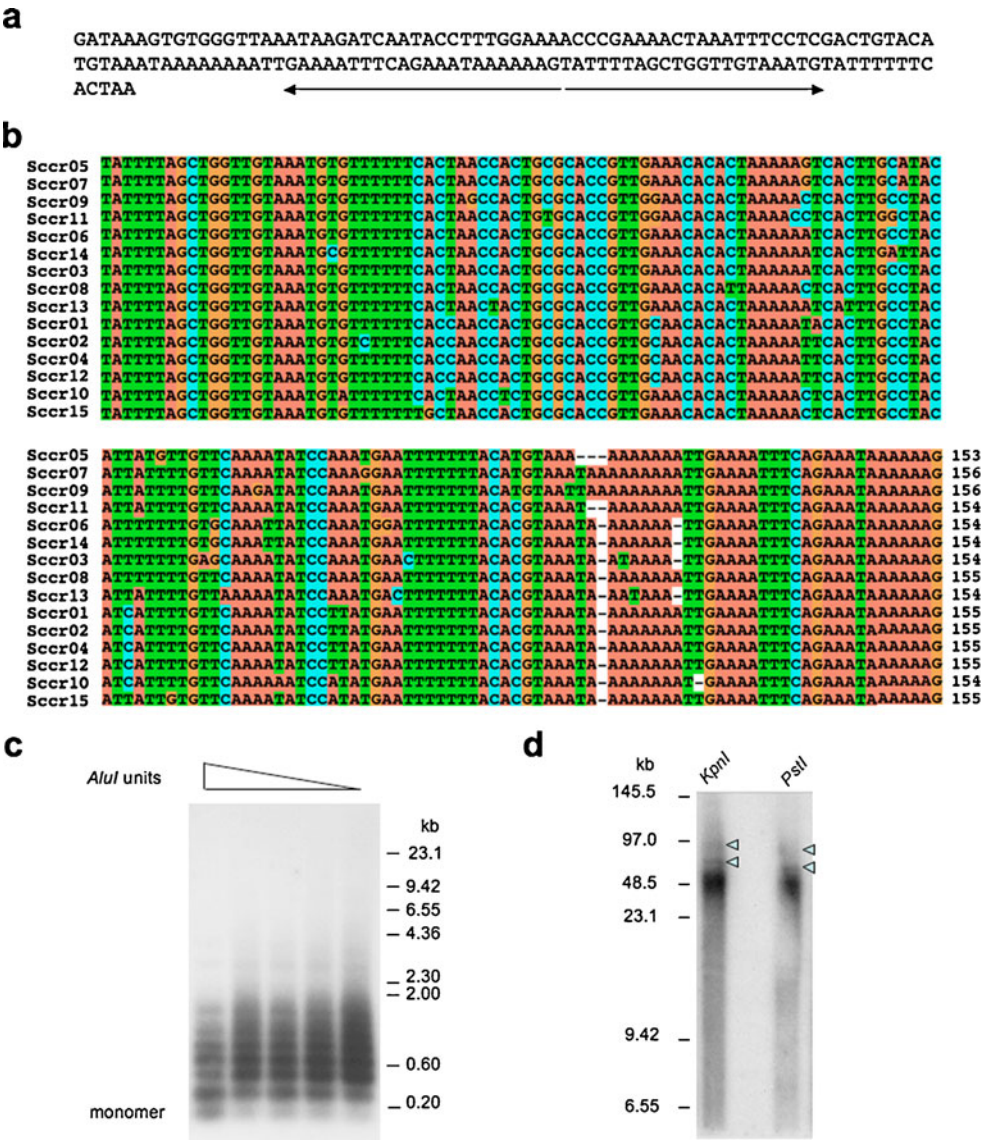


Fig. 5 Sequence alignment and genomic organization of the Sccr repeats. **a** Sequence of G2 (the X.01G2 insert). The PCR primers derived from the region are underlined with *two arrows*. **b** Multiple sequence alignment of 15 Sccr repeat units; T (*green*), A (*red*), G (*orange*), and C (*blue*). **c** Southern blot of *AluI*-partially digested genomic DNAs hybridized with P^{32}-labeled Sccr01 DNA after separation in a conventional 0.8% agarose gel. **d** Southern blot of *KpnI* and *PstI* digested genomic DNAs hybridized with P^{32}-labeled Sccr01 DNA after fractionation on a 0.8% agarose gel run at 150 V for 20 h with a pulse time of 14 s. The largest bands are indicated with *arrowheads*

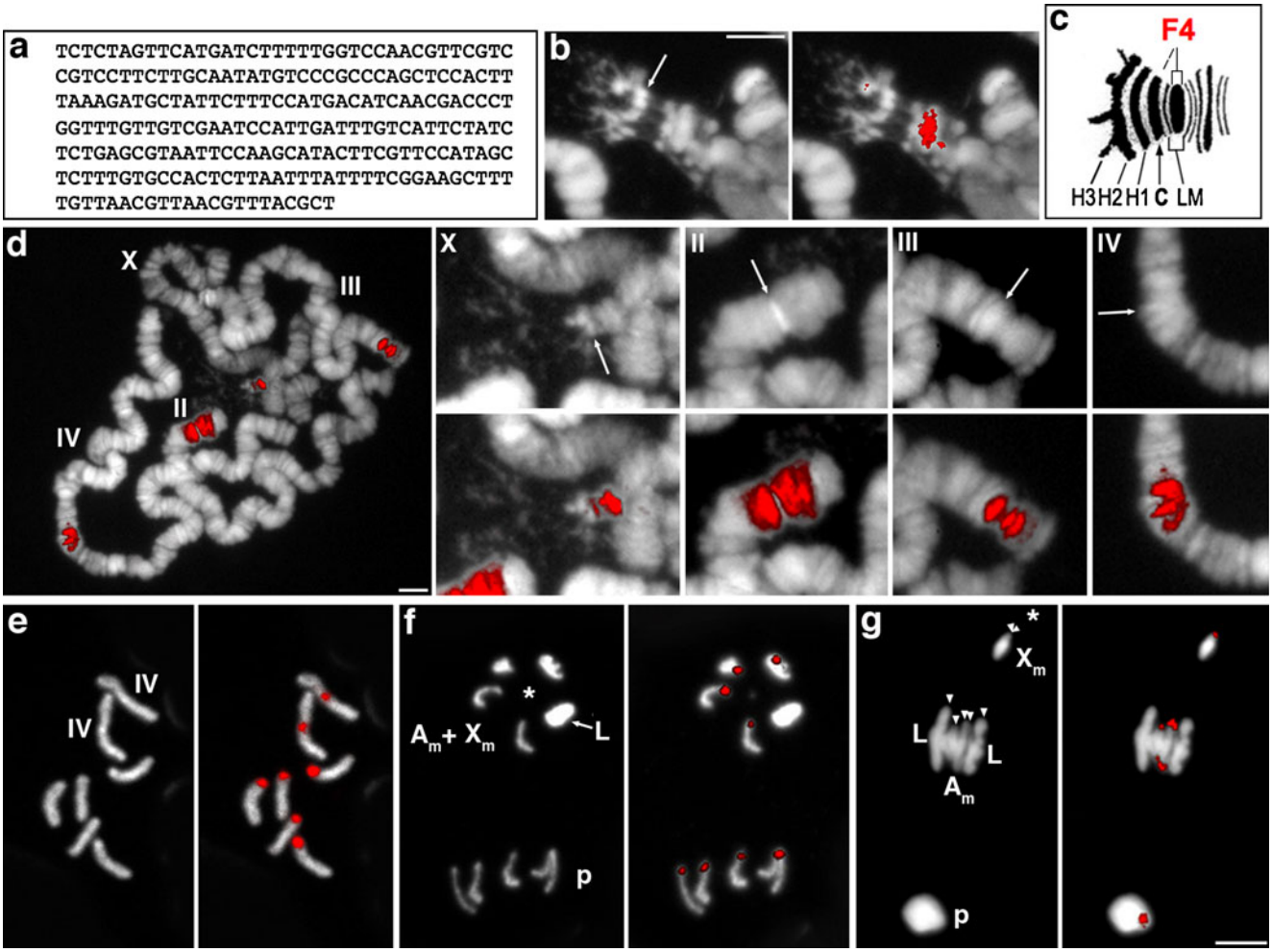
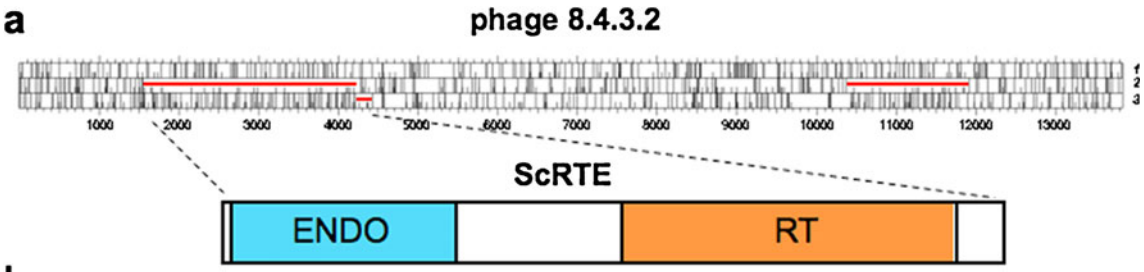


Fig. 6 F4 in situ hybridization in *S. coprophila* polytene chromosomes (**b**–**d**), neuroblast mitotic chromosomes (**e**), and spermatocytes at meiosis I (**f**) and meiosis II (**g**). **a** Sequence of F4 (the X.01F4 insert). **b** A detailed view of the X chromosome centromeric end showing the location of F4 with respect to the centromere (*arrow*). **c** Schematic diagram of the X chromosome centromeric end (Gabrusewycz-Garcia 1964; Crouse et al. 1977) showing the location of F4 (*red*) at the LM and, to a minor extent, at the centromere region. **d** F4 signals are present at the pericentromeric region of all chromosomes (*arrows*). **e** Neuroblast mitotic chromosomes showing centromeric F4 signals in all chromosomes; metacentric chromosomes (IV) are indicated. **f** At the end of meiosis I, all maternal chromosomes plus L chromosomes associate with the polar complex while paternal chromosomes segregate to a cytoplasmic bud to be eliminated. All chromosomes, except the L chromosomes, exhibit F4 centromeric staining. **g** Early anaphase II showing F4 signals at the centromeric regions (*arrowheads*) of all chromosomes except the L chromosomes. The F4 signals in p correspond to the centromeres of the eliminated paternal chromosomes. X_m maternal X chromosome, A_m maternal autosomal set, *p* paternal chromosomes, *L* germline-limited chromosomes; *asterisk* indicates the deduced position of the polar complex. *Scale bar* 10 μm

chromosome, in addition to the centromere, G2 hybridized weakly to H1 (see Fig. 4b, c). The centromere labeling was clearly observed in all mitotic (Fig. 4e) and meiotic chromosomes (Fig. 4f).

Given the centromeric location of G2, we decided to find out whether G2 might be a part of a tandem repeat. To test this possibility, we utilized a PCR-based approach to obtain complete units using genomic DNA and divergent primers from the DNA sequence of G2 (see Fig. 5a). After size fractionation of the PCR-products, by agarose gel electrophoresis, a ladder with monomers and multimers of a unit of approximately 150 bp was observed, confirming the presence of centromeric tandem

Fig. 7 Characteristics and phylogenetic analysis of the ScRTE elements. **a** Three-frame ORF map of the insert of phage 8.4.3.2. The location of the two ScRTE elements is indicated with *red lines*. A diagram of the structure of an entire ScRTE element is shown at the bottom. The AP endonuclease (ENDO) and RT domains within the ORF are indicated by *blue* and *orange*, respectively. **b** Sequence of the 955-amino-acid ORF. The AP endonuclease and RT domains are highlighted in *blue* and *orange*, respectively. **c** Phylogenetic relationship of non-LTR retrotransposons including the ScRTE element. The tree was generated using a neighbor-joining method. The RT domains of the CRE elements were used as outgroups. For the new amino acid alignment, we have used the previous sequence alignment EMBL: DS36752 (Malik et al. 1999), but including only elements from CR1, R1, R2, L1, jockey, RTE, and CRE clades with our ScRTE element. Bootstrap values are given as percentage numbers. The name of the elements and clades are given to the *right*. An amino acid divergence scale is shown at the *top*



b

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MLKTFKRSHNELYIATYNARTLSSRQKMQEMEEELEKIKWDVVGVSEVRKPGEECLKFQSGHTFFYRGSDSQMLMHGVGL
FINKRWSDRIIHTKSISDRVIYVSLKINNRYSVKLIQVYAPTSTHDDEEVERFYEDVEKALDENPSHYQYLIGDFNAKLG
KREEDSEVSVGSFSNDQRNERGNTLLNFLQQHNLYAMNSFFAGKPQRKWTWASADGVTKNEIDYIITGCKSTVKNVTVLN
NFSTGSDHRMVRARVTLNTRFQRSQLVQKSERIDVQRLYTQNEEFATKMTAGLDNVNNQCETLDELNTKIVDTIMGFMKS
KCKSVQGKESKLSRETLDLIASRAELVKNGGRDSVEYRNLSKSINKARRSDERKYNVQLAQNIIEANCNMKVLRRKMTNA
KKEIFKLRDKTGTIQTDRNAILNIATEFYENLFSSARQSPTEETEDRPIIRNVGSEDMPDITVDEVKAAVAEMKNKKSPG
EDGVPVEAIKLGGDSLLKAITALFNQCLQWEEVPEAWENAVITLLHKKGDITKLENYRPISLLSTLYKLFMKIITKRNTN
KFDFYQPVEQAAFRSGFSTNDHLQVMRTLIEKCREYNIDIVLLFIDFEKAFDSVETWSILDALDECRVDSRYSNTIRYVY
KNATSCIKLHKSTEKFRIGRGVRQGDTISPKLFTAILQSIFRKLNWSKMGIKTNGEYLSNLRFADDIVPIAANLGQAQLM
LQQLSEEASKVGLKMNLSKTKVMTNIGDDREIKIGDTVIERVDSYVYLGHKLKLGLDNQTAEIRRRIGLAWAAFGKLRLI
FKSKMNNSLKRKVFDTCVLPVLTYGAETLTLTKASEEKLRVTQRAMERSMLGITLRDRMTNQWIRQQTRVVDVMERIASL
KWSWAGHIARRTDERWTKKIMNWRPYKRRAIGRPPERWTNGIKNIAGTNWQQMAMDRTKWKEVGEAYIQQWIETG
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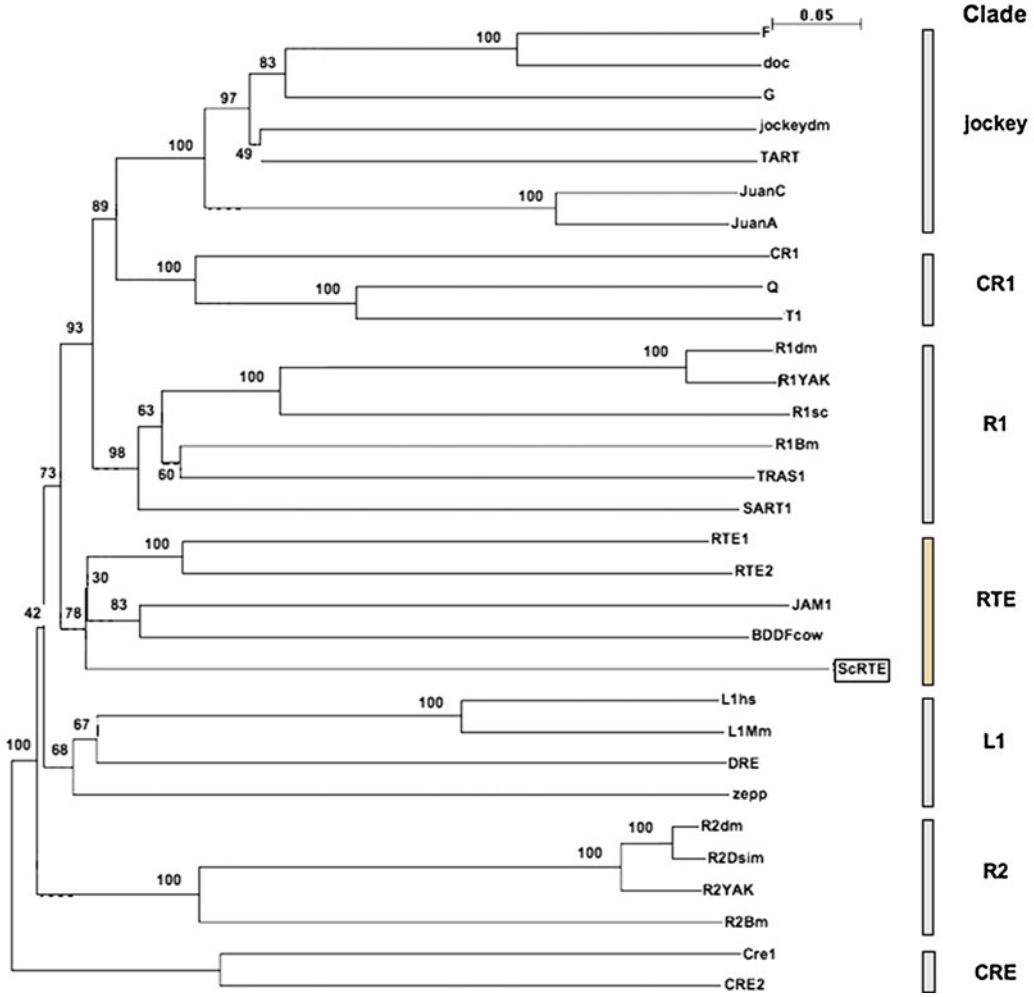
c



repeats. Fifteen PCR clones were sequenced and the sequence alignment of the repeats showed a consensus length of 155 bp (Fig. 5b). We have named this highly repeated sequence Sccr (for *Sciara coprophila* centromeric repeat). The overall sequence is AT rich (72%) and presents a high degree of sequence similarity between

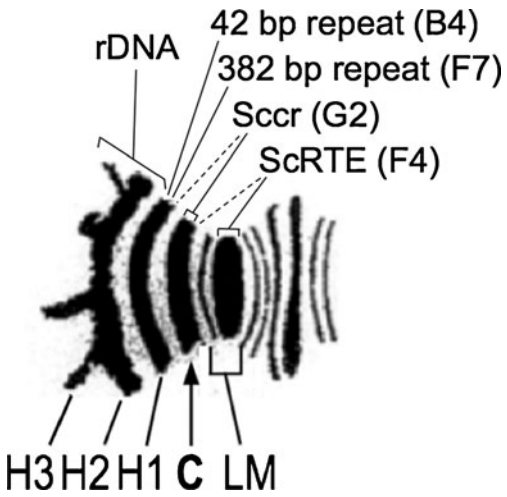


Fig. 8 Probable map order of the rDNA, the 42 bp repeats, the 382 bp repeats, the Sccr repeats, and the ScRTE elements in centromeric X heterochromatin. This heterochromatin was subdivided by translocations into three blocks (H1, H2, and H3) by Crouse et al. (1977). The *arrow* indicates the position of the centromere in the X chromosome centromeric end diagram (Gabrusewycz-Garcia 1964; Crouse et al. 1977)

repeats (around 95% identity). The differences between the Sccr units are scattered single-base changes and small deletions (Fig. 5b). The high AT content of Sccr suggests that this repeat is different from the satellite DNA previously described by Abbott et al. (1981).

Complex satellite DNAs have also been found at all the centromeres of the lower dipteran *Chironomus pallidivittatus* (Rovira et al. 1993). However, in the higher dipteran *Drosophila melanogaster*, there is not a common centromeric sequence (Abad et al. 1992; Lohe et al. 1993), and only the X chromosome and Y chromosome have complex centromeric satellites (Lohe et al. 1993; Abad et al. 2000; Agudo et al. 2000; Méndez-Lago et al. 2009). This fact has hindered the *D. melanogaster* centromere analysis over the years, so the genomic sequence organization of each centromere remains incomplete.

To investigate the physical organization of Sccr repeats, we first digested genomic DNA with the restriction endonuclease *AluI*, which cuts the repeat at a single site. The digested DNA was fractionated by agarose gel electrophoresis, transferred to filter, and hybridized with radioactive Sccr01 DNA. Partial digestions with *AluI* showed ladders of bands, where the size of the multimers corresponded to multiples of the155-bp unit length (Fig. 5c). This analysis revealed the presence of homogeneous tandem arrays of Sccr repeats in the genome. Moreover, to have an estimation of repeat array sizes, restriction enzymes having 6 bp recognition sites and lacking sites in the repeat were used. Digestions with *KpnI* and *PstI* gave rise to bands in the range of 30–80 kb (Fig. 5d), suggesting the presence of satellite blocks as large as 80 kb.

Pericentromeric RTE elements in *S. coprophila*

A BlastX search of the GenBank database with the X.01F4 sequence (Fig. 6a) revealed strong similarity with a portion of the reverse transcriptase of non-LTR retrotransposons of the RTE clade. RTE elements are autonomous retrotransposons broadly distributed in animals that contain a single open reading frame (ORF) encoding a protein with endonuclease and reverse transcriptase activity (Malik and Eickbush 1998; Malik et al. 1999).

FISH with F4 on polytene chromosomes showed very strong labeling at the pericentromeric regions (Fig. 6b–d), with symmetrical and asymmetrical signal distributions. Thus, the labeling was preferentially at the "land mark" next to the centromere of the X chromosome, at the short arm of the chromosome III, and at both arms of chromosomes II and IV (Fig. 6d). The "land mark" (LM) is the name given by Crouse (1977) to the prominent double band at the right of the X centromere (see Fig. 6c). In these in situ hybridization experiments, it was possible to distinguish that F4 hybridized strongly to the distal band of the LM and weakly to the centromere region (Fig. 6b–d). The pericentromeric labeling was also observed in mitotic and meiotic chromosomes (Fig. 6e, g), but not in L chromosomes (Fig. 6f, g).

In order to isolate a full-length copy of the putative RTE, we screened a *S. coprophila* genomic library in λ DASH II with F4 as the probe. The sequence of the positive phage 8.4.3.2 contained an entire or almost entire 2,917-bp element with a frame shift mutation and a 5′ truncated 1,495-bp element (Fig. 7a). These elements have an unusually short 3′ untranslated region (AAAAGAAGAA GAAGAAGAAGA) and are flanked by different target-site duplications, such as the members of the RTE clade (Malik and Eickbush 1998). The full-length element encodes a 955 amino acid ORF containing both apurinic–apyrimidic endonuclease and reverse transcriptase domains (Fig. 7a, b). Finally, a phylogenetic analysis has identified this pericentromeric element, that we name Sc-RTE, as a member of the RTE clade (Fig. 7c).

In conclusion, as summarized in Fig. 8, we here provide the first localization of repetitive DNA sequences in the heterochromatin of *S. coprophila* X chromosome. These findings are consistent with reports coming from different organisms, where islands of transposons embedded in large regions of simple and/or complex sequence repeats contribute to a common centromere organization (Schueler et al. 2001; Sun et al. 2003; Nagaki et al. 2004; Méndez-Lago et al. 2009).

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"Fosforilación de la histona H3 y no-disyunción del cromosoma X materno durante la meiosis masculina en *Sciara*"

Resumen

El diptero *Sciara* presenta una segregación cromosómica altamente anómala ya que durante la meiosis masculina tienen lugar la eliminación del complemento cromosómico paterno completo así como la no-disyunción del cromosoma X materno. Durante la meiosis I, en la que los cromosomas paternos son eliminados, la segregación de los cromosomas maternos está dirigida por un huso monopolar. En el transcurso de la meiosis II, mientras los autosomas maternos segregan normalmente, el cromosoma X materno permanece asociado al polo generado en meiosis I y no realiza la disyunción de las cromátidas. La actividad centromérica del cromosoma X está regulada por un *locus* que actúa en *cis* y que se localiza en la heterocromatina adyacente al centrómero. Mediante experimentos de inmunofluorescencia en espermatocitos de *Sciara ocellaris y S. coprophila*, se ha llevado a cabo el análisis de los patrones de fosforilación de la histona H3 en las serinas 10/28 y las treoninas 3/11 durante las distintas fases de la meiosis masculina. Los resultados obtenidos han permitido concluir que tanto el nivel de condensación, como el patrón de fosforilación de la histona H3, difieren entre cromosomas de diferente origen parental durante la eliminación del complemento paterno en meiosis I. Por otra parte, durante la meiosis II el cromosoma X materno se diferencia del resto de cromosomas maternos en que su región centromérica no está fosforilada en ninguno de los cuatro residuos de la histona H3 estudiados. Los resultados obtenidos constituyen la primera prueba experimental que relaciona la falta de fosforilación de la histona H3 en la región centromérica del cromosoma X materno con la no-disyunción meiótica de dicho cromosoma en *Sciara*. Nuestros resultados apoyan fuertemente que la falta de H3 fosforilada está directamente implicada en la inactivación del centrómero del cromosoma X durante la transición meiosis I-II.

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Histone H3 phosphorylation and non-disjunction of the maternal X chromosome during male meiosis in sciarid flies

M. Carmen Escribá[1,*], M. Cecilia Giardini[2,*] and Clara Goday[1,‡]

[1]Centro de Investigaciones Biológicas, Departamento de Proliferación Celular y Desarrollo, CSIC, Ramiro de Maeztu 9, 28040 Madrid, Spain
[2]Instituto de Genética "Ewald A. Favret", INTA-Castelar, C. C. 25-1712, Argentina

*These authors contributed equally to this work
‡Author for correspondence (claragoday@cib.csic.es)

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Summary

An extremely unorthodox method of chromosome segregation is found in sciarid flies (Diptera, *Sciaridae*), where at male meiosis, the whole paternal complement is eliminated and the maternal X chromosome undergoes non-disjunction. At meiosis I, a monopolar spindle directs the segregation of maternal chromosomes to the single pole, whereas paternal chromosomes are discarded. At meiosis II, although maternal autosomes segregate normally, the X chromosome remains undivided. A *cis*-acting locus within the heterochromatin proximal to the centromere is known to regulate X centromere activity. By immunofluorescence analysis in spermatocytes from *Sciara ocellaris* and *Sciara coprophila*, we investigated histone H3 phosphorylation at Ser10, Ser28, Thr3 and Thr11 during male meiosis. We found that chromosome condensation and H3 phosphorylation patterns differ between chromosomes of different parental origin at the time of paternal set elimination. Importantly, at meiosis II, the maternal X chromosome differs from the rest of the chromosomes in that its centromeric region does not become phosphorylated at the four histone H3 sites. We provide here the first evidence linking the under-phosphorylated H3 status of the X chromosome centromeric region with its meiotic non-disjunction in sciarid flies. Our findings strongly support the idea that the deficiency in local H3 phosphorylation inactivates the X centromere at the transition from meiosis I to meiosis II.

Key words: H3 phosphorylation, *Sciara*, Meiosis, Chromosome non-disjunction, Chromosome elimination

Introduction

A classical example of unorthodox chromosome segregation is found in sciarid flies (Diptera, *Sciaridae*) where the selective elimination of paternal chromosomes takes place at different times during development (Metz, 1925; Metz, 1926b; Metz, 1933) (reviewed in Gerbi, 1986; Goday and Esteban, 2001). One of the most complex and bizarre chromosome behaviours occurs in sciarid male meiosis, where the whole paternal chromosome complement is discarded so that only maternally derived chromosomes are included in the sperm nucleus. An additional exceptional feature of male meiosis is the occurrence of non-disjunction of the maternal X chromosome (X_m), which determines the characteristic 3X constitution of the zygote in sciarids (reviewed in Gerbi, 1986; Goday and Esteban, 2001). Since the early findings on the chromosome cell cycle of sciarids, the mechanisms that eliminate paternal chromosomes at meiosis I and non-disjunction of X_m chromosome at male meiosis II have been the subject of several studies (reviewed in Gerbi, 1986; Goday and Esteban, 2001). In Fig. 1 we summarise the most relevant chromosomal events occurring during male meiosis that are common to sciarid flies. As shown (Fig. 1A), at the first meiotic division there is no pairing of homologous chromosomes at prophase and the chromosomes do not align in a metaphase-like array (Metz, 1925; Metz, 1926a; Metz, 1926b; Fuge, 1994). Instead, they proceed directly from prometaphase to an 'anaphase-like' stage (Fig. 1B). This atypical behaviour is accompanied by the formation of a monopolar spindle generated from a single polar complex that contains 'giant centrioles' surrounded by a large amount of pericentriolar material, from which numerous microtubules radiate (Kubai, 1982; Fuge, 1994; Esteban et al., 1997). During the anaphase-I-like stage, the monopolar spindle directs the segregation of maternal chromosomes to the single pole, whereas paternal chromosomes move in the opposite direction into a cytoplasmic bud to be discarded later (Metz, 1925; Smith-Stocking, 1936). Importantly, cytological and ultrastructural data indicate that the two parental sets of chromosomes occupy distinct nuclear compartments in germ nuclei from the initial stages of development until the occurrence of meiosis (Rieffel and Crouse, 1966; Kubai, 1982; Kubai, 1987; Goday and Esteban, 2001; Goday and Ruiz, 2002). Moreover, the precocious intranuclear segregation of the maternal chromosomes with respect to the paternal ones is considered to be essential in determining which chromosomal set will be lost during male meiosis I (Kubai, 1987; Goday and Esteban, 2001).

The outstanding feature of meiosis II is the different kinetic behaviour of the maternal X chromosome with respect to maternal autosomes (Metz, 1925; Crouse, 1943) (reviewed in Esteban et al., 1997). By metaphase II (Fig. 1C), whereas maternal autosomes align in a typical metaphase plate, the non-disjoining X_m chromosome does not move away from the polar complex formed at meiosis I. Importantly, microtubules radiating from the single pole interact with the X_m centromere and maintain the non-disjunction of this chromosome throughout meiosis (Esteban et al., 1997). Finally, at anaphase II (Fig. 1D,E), the autosomal chromatids segregate to opposite poles in the conventional manner by means

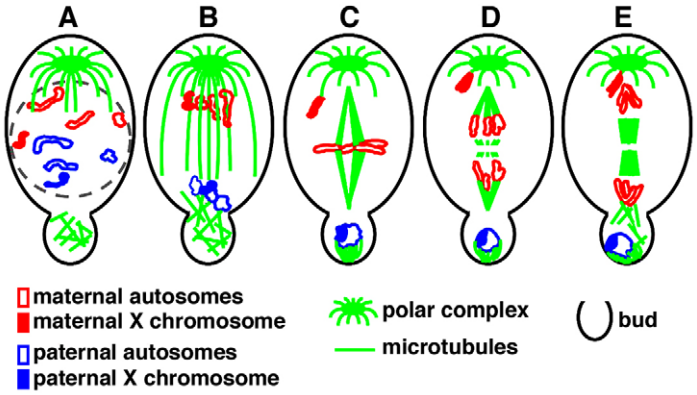

Fig. 1. Scheme summarising the most relevant chromosomal events of male meiosis in *S. ocellaris*. (**A**) Prophase I. Prophase chromosomes do not pair. Maternal and paternal chromosomes display separate arrangement within the spermatocyte nucleus. A monopolar spindle is formed and non-spindle microtubules are generated in a cytoplasmic bud region of the spermatocyte. (**B**) Anaphase-I-like stage. Maternal chromosomes segregate towards the spermatocyte single polar complex while the paternal set move into the bud. (**C**) At metaphase II, whereas the maternal X chromosome remains attached to the polar complex developed in meiosis I, maternal autosomes align at the equatorial plate and an asterless bipolar second meiotic spindle is developed. (**D**,**E**) At anaphase II, whereas the maternal autosomal chromatids segregate towards opposite directions in a conventional manner, the maternal X chromosome remains undivided and attached to the single pole of the first meiotic spindle that persists throughout meiosis (D). The upper maternal autosomal chromatid group joins the X chromosome to constitute the future sperm nucleus, whereas the opposite maternal chromatid group (X-null chromatid set) is captured by bud microtubules and, along with the paternal set, is eliminated from the spermatocyte by a bud excision process (E) (modified from Goday and Esteban, 2001).

of an asterless bipolar second meiotic spindle (Metz, 1926a; Abbott and Gerbi, 1981; Esteban et al., 1997).

The genetic control of X_m chromosome segregation at male meiosis was examined in *Sciara coprophila*, where three heterochromatic blocks proximal to the centromere contain rDNA sequences (Gerbi and Crouse, 1976; Crouse, 1977; Crouse et al., 1977; Crouse, 1979). A *cis*-acting locus, the *controlling element* (*CE*), regulating X-centromere activity was identified in the middle heterochromatic block (Crouse, 1960; Crouse, 1977; Crouse, 1979; Gerbi, 1986). Its translocation to an autosome provokes non-disjunction of the recipient autosome, whereas the X chromosome lacking the *CE* segregates normally (Crouse, 1979). In view of this, it has been hypothesised that the *CE* inhibits normal centromeric function in the X_m chromosome (Gerbi, 1986). However, how this occurs and which specific DNA sequences are involved remains undetermined.

In the present work, we have re-examined the process of male meiosis in *Sciara ocellaris* and *S. coprophila* in an attempt to further understand the cellular mechanisms leading to both paternal chromosome elimination and X_m chromosome non-disjunction. With this in mind, we decided to analyse conserved chromatin modifications involved in normal chromosome segregation during mitosis and meiosis. A good candidate was that of histone H3 phosphorylated at four N-terminal residues, Ser10, Ser28, Thr3 and Thr11 (reviewed in Nowak and Corces, 2004; Xu et al., 2009). Cell-cycle-dependent phosphorylation of histone H3 (H3-*P*) occurs in most eukaryotes and high levels of H3-*P* constitute a conserved mark of mitotic cell division. As a general rule, condensed metaphase chromosomes attain high levels of phosphorylated histone H3 and upon exit of mitosis or meiosis, a global dephosphorylation of histone H3 occurs. Moreover, an increasing amount of data coming from different systems support the idea that the four H3-*P* forms are coordinated both in space and time during mitosis and meiosis (Xu et al., 2009).

Phosphorylation of H3S10 (H3S10-*P*) (Gurley et al., 1978; Wei et al., 1998; Hsu et al., 2000; Giet and Glover, 2001) and of H3S28 (H3S28-*P*) (Goto et al., 1999), are carried out by the mitotic kinase Aurora B that is a component of the chromosomal passenger complex (CPC) (Giet and Glover, 2001; Ruchaud et al., 2007). Both H3-*P* modifications are highly conserved among eukaryotes and are crucial for higher-order chromatin compaction during mitosis and meiosis (reviewed in Hsu et al., 2000; Nowak and Corces, 2004). The temporal correlation of H3S10-*P* with chromosome condensation during cell cycle progression was first demonstrated in *Tetrahymena* using antibodies against H3S10-*P* (Wei et al., 1998). Accordingly, mutation of the site H3S10 in *Tetrahymena* disrupts proper chromosome condensation and segregation both at mitosis and meiosis (Wei et al., 1999). A number of studies have shown that in mammals, the cell-cycle-dependent phosphorylation of H3S10 and H3S28 begins at the pericentromeric chromosome regions and spreads throughout the chromosomes during the G2–M phase transition (Goto et al., 1999; Hsu et al., 2000). A similar distribution of H3S10-*P* linked to chromosome condensation has also been found in flies where depletion of Aurora B provoked deficiencies of chromosome condensation at mitosis (Giet and Glover, 2001). Moreover, in *Drosophila* spermatocytes undergoing the first meiotic division, metaphase I chromosomes show prominent H3S10-*P* signals that decrease substantially at anaphase and telophase (Krishnamoorthy et al., 2006).

Phosphorylation of H3T3 (H3T3-*P*) by the kinase Haspin (Dai et al., 2005) and of H3T11 (H3T11-*P*) by Dlk/ZIP (Preuss et al., 2003) were found to temporally associate with mitosis in turkey and mammalian cells (Polioudaki et al., 2004; Dai et al., 2005). The timing of H3T3 phosphorylation and dephosphorylation is similar to that of H3S10-*P*, although the strongest presence of H3T3-*P* at the inner centromeric regions of the chromosomes suggested a more direct role of H3T3-*P* in regulation of kinetochore assembly and functional activity (Dai et al., 2005). In this regard, recent findings in mammalian cultured cells revealed for the fist time an essential functional role of H3T3 phosphorylation at the centromere site (Wang et al., 2010). Phosphorylated H3T3 is crucial for the

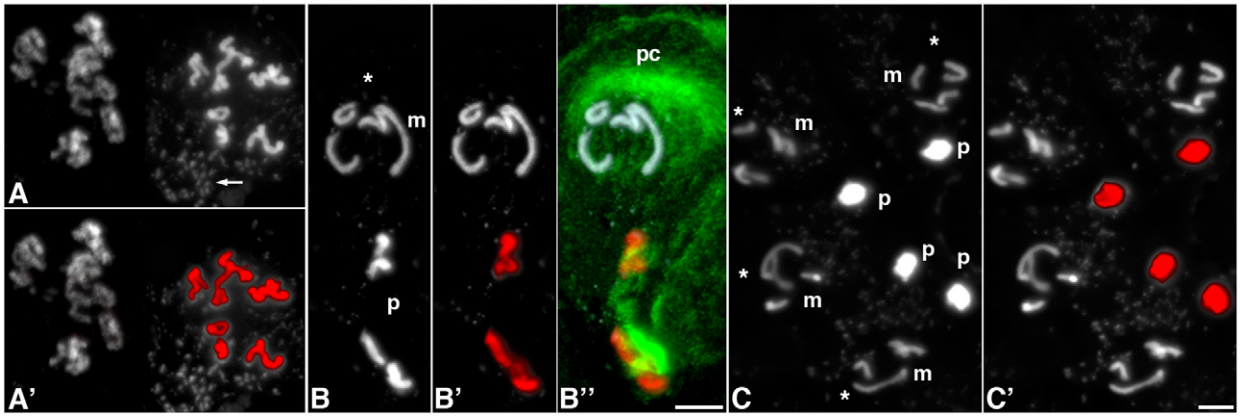

Fig. 2. Distribution of histone H3S10-*P* in *S. ocellaris* spermatocytes in the first meiotic division. (**A–C′**) Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole (polar complex or 'pc' visualised by anti-tubulin staining). (A′–C′) Indirect immunolabelling with the H3S10-*P* antibody and merged images where antibody staining is in red. (B″) Double-immunolabelling with H3S10-*P* antibody (red) and anti-tubulin antibody (green). (A,A′) Prophase nuclei undergoing chromosome condensation; H3S10-*P* labelling is detected in all chromosomes as chromatin condensation increases (nucleus on the right in A′); arrow indicates mitochondria. (B–B″) Anaphase-like stage where the maternal four chromosomes facing the pole (pc in B″) appear less condensed than the paternal set segregating towards the bud; antibody labelling is restricted to the four paternal chromosomes (B′). (C,C′) End of meiosis I; a partial view of a cyst showing four spermatocytes arranged radially with respect to the lumen of the cyst; maternal chromosomes remain at the pole and lack H3S10-*P* staining; paternal chromosomes, tightly grouped, exhibit H3S10-*P* labelling. Scale bars: 10 µm.

localisation of the CPC at centromeres and for the function of Aurora B during mitosis (Wang et al., 2010). Moreover, as shown in *Xenopus* extracts, defects in the recruitment of the CPC to the chromosomes and in the activation of Aurora B leads to alterations in spindle assembly around chromosomes (Kelly et al., 2010).

In this work we have analysed the distribution of the four H3-*P* forms in spermatocytes of *S. ocellaris* and *S. coprophila* during the meiotic divisions. We provide here a comparative location and timing description of each of the H3-*P* modification during both meiotic divisions. From this analysis, we found that at meiosis I, at the time of paternal chromosome elimination, chromosome condensation and H3 phosphorylation patterns differ between chromosomes of different parental origin. Moreover, and importantly, we show that non-disjunction of the X_m chromosome during meiosis II correlates with the lack of the X_m centromeric region to undergo phosphorylation of histone H3. Our findings strongly indicate that the deficiency in H3 phosphorylation inactivates X_m centromere at the transition from meiosis I to meiosis II. The results also suggest that the *CE* induces a local deficiency of H3 phosphorylation in both maternal and paternal X chromosomes.

Results

The chromosomal complement in *S. ocellaris* premeiotic germ nuclei is that of eight chromosomes: three pairs of autosomes (two acrocentric and one metacentric) and one pair of X chromosomes (acrocentric). In *S. coprophila*, additional germline-limited 'L' chromosomes are present in a variable number (1–4). L chromosomes are metacentric and, in contrast to the regular chromosomal component, are mostly heterochromatic.

Distribution of phosphorylated H3S10 in *S. ocellaris* spermatocytes during meiotic division

We performed the immunodetection of H3S10-*P* in *S. ocellaris* in male meiosis I when the elimination of the whole paternally inherited complement takes place. As mentioned, in *Sciara* males, homologous chromosomes do not pair at prophase nor do they align in a metaphase-like array. Instead, they proceed directly from prophase to an 'anaphase-like' stage (Gerbi, 1986; Esteban et al., 1997). Intranuclear H3S10-*P* labelling was detected in all chromosomes at prophase stage when a significant degree of chromosome condensation was achieved (Fig. 2A,A′, nucleus on the right). Fig. 2B–B″ shows an example of a DAPI-stained first 'anaphase-like' figure, together with the corresponding monopolar first meiotic spindle evidenced by anti-tubulin staining. Immunolocalisation of H3S10-*P* (Fig. 2B′) revealed that the antibody associates exclusively to paternal chromosomes (p, segregating towards the cytoplasmic bud), whereas the maternal set (m, already near to the polar complex) is devoid of staining. In addition, the chromosomes that displayed H3S10-*P* signals (paternal set) exhibited a considerably higher degree of condensation with respect to the unstained ones (maternal set), as seen by DAPI staining (Fig. 2B). H3S10-*P* staining differences between the two parental chromosome groups could be observed until the end of the 'anaphase-like' stage (Fig. 2C,C′), where paternal chromosomes, usually tightly grouped and highly fluorescent with DAPI, are eliminated in buds into the lumen of the cyst. From these results, we concluded that in the 'anaphase-like' stage of meiosis I, the two chromosomal groups differ in H3S10 phosphorylation and in chromosome condensation levels, with the paternal chromosomes phosphorylated and more highly condensed than the maternal homologues. Before this stage, as shown in prophase nuclei (Fig. 2A,A′), both maternal and paternal chromosomes had transiently achieved a similar degree of condensation and significant levels of H3S10-*P*. Thus, during meiosis I, paternal chromosomes segregating towards the spermatocyte bud remain condensed and H3S10 phosphorylated, whereas the maternal set associated with the polar complex appears to some extent decondensed and under-phosphorylated at H3S10.

We next examined H3S10-*P* distribution during the second meiotic division. The transition from meiosis I to meiosis II is clearly marked by H3S10 phosphorylation of maternal

chromosomes together with increasing chromosome condensation (Fig. 3A,A′). At this phase, we frequently observed that one of the maternal acrocentric chromosomes was devoid of H3S10-*P* signals at one chromosomal end (Fig. 3A,A′, arrow in m). In some of the spermatocyte squashes, it was also possible to detect a small un-stained chromosomal region in the bulk of the

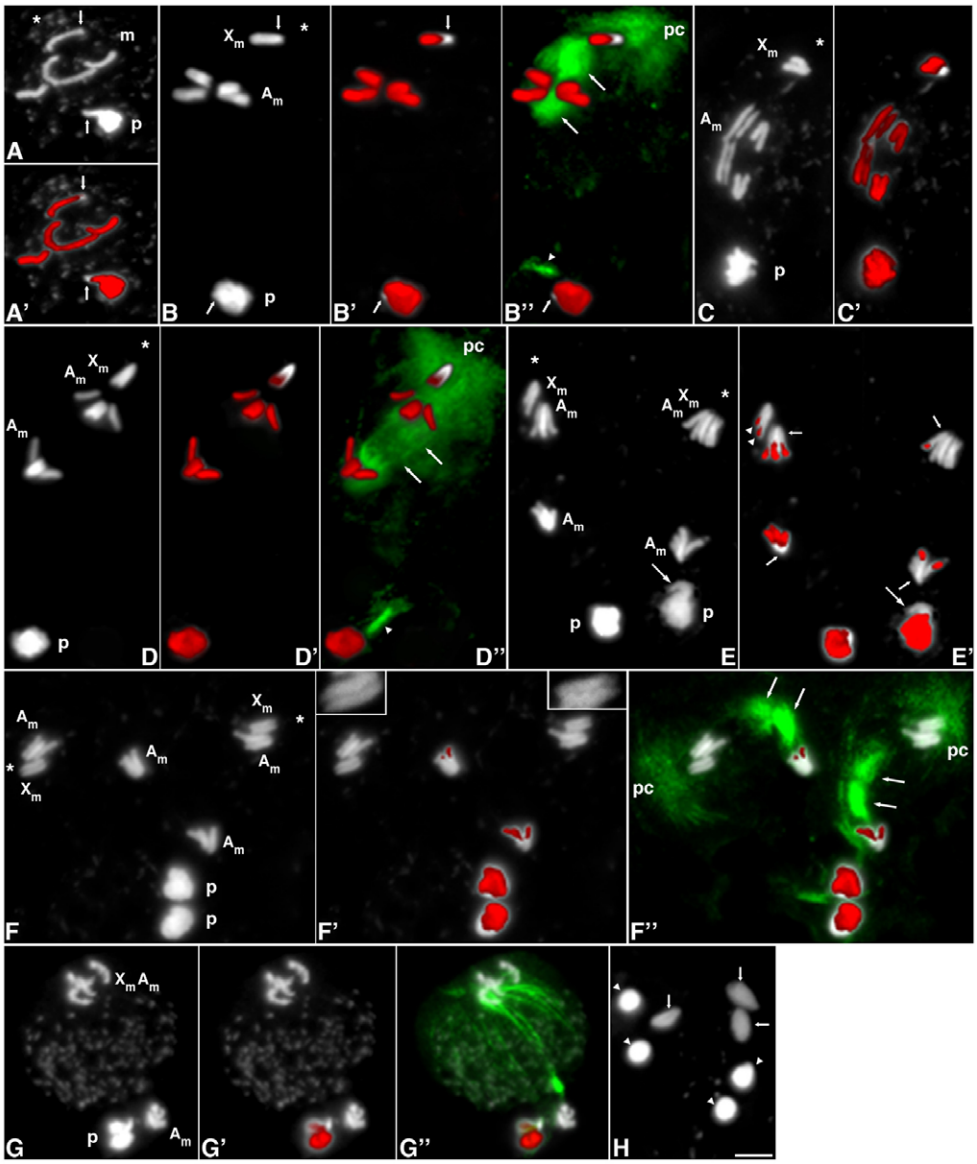

Fig. 3. Distribution of histone H3S10-*P* in *S. ocellaris* spermatocytes undergoing the second meiotic division. (**A–H**) Chromatin DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. (A′–G′) Indirect immunolabelling with H3S10-*P* antibody and merged images where antibody staining is in red. (B″,D″,F″,G″) Double-immunolabelling with H3S10-*P* antibody (red) and anti-tubulin antibody (green). (A,A′) Meiosis I–II transition; maternal and paternal chromosomes exhibit H3S10-*P* staining except at one end of one of the acrocentric maternal chromosomes plus at a protruding piece of paternal chromosomes (arrows in m and p). (B–B″) Metaphase II; X_m chromosome is H3S10-*P* labelled except at the chromosome end containing the centromere, as seen by its association to polar microtubules (B″); maternal autosomes at the metaphase plate (two acrocentric and one metacentric, A_m in B) are entirely decorated by the antibody (B′); arrows in (B″) indicate the asterless second meiotic spindle and arrowhead denote non-spindle bud microtubules. In the lower part of (B,B′) arrows indicate a small region of paternal chromatin lacking antibody staining. (C,C′) Early anaphase II where maternal autosomes are decorated by the antibody. (D–D″) Anaphase II progression showing that the separated maternal autosomes are entirely labelled (D,D′); in D″, arrows indicate the second meiotic spindle midzone and arrowhead denotes non-spindle bud microtubules. (E,E′) Two nearby spermatocytes showing the maternal autosomal chromatids moving closer to the X chromosome; antibody staining is progressively reduced starting from the centromeric regions (arrows in E′); X_m chromosome also shows a reduction of H3S10-*P* signals in both chromatids (arrowheads in E′); long arrows in E,E′ denote a non-stained region protruding from the paternal chromatin bulk. (F–F″) Two nearby spermatocytes in late anaphase where the maternal chromatids have reached the X chromosome at the polar complex. (F′) H3S10-*P* staining is restricted to the tips of the A_m chromatids furthest from the direction of movement towards the bud and to paternal chromosomes; insets in F′ show enlarged X_m chromosomes where sister chromatids appear are totally separated; arrows in F″ indicate the two halves of the second meiotic spindle. (G–G″) Spermatocyte at the end of meiosis II; (G) position of the future sperm-nucleus maternal components (X_m+A_m) and of the discarded maternal X-null chromatid set (A_m) plus paternal chromosomes (p). (G′) Antibody labelling is restricted to paternal chromosomes. (H) Spermiogenesis; both spermatid nuclei (arrows) and eliminated chromatin (arrowheads) in the buds are devoid of H3S10-*P* staining. Scale bar: 10 μm.

eliminated paternal chromosomes (Fig. 3A,A′, arrow in p). The analysis of metaphase II (Fig. 3B–B″). where the non-disjoining X_m chromosome remains attached to the polar complex whereas the maternal autosomes (A_m) align in a metaphase plate, permitted us to identify the X_m chromosome as that lacking H3S10-*P* signals at one chromosome end. Moreover, the X_m chromosome end devoid of the H3S10-*P* label was the one containing the centromere, as seen by its typical spatial location and association with the polar complex microtubules visualised by anti-tubulin staining (Fig. 3B″, pc). During anaphase II (Fig. 3C–F′), H3S10-*P* labelling of maternal autosomes persists until sister chromatids are completely detached (Fig. 3D–D″). At late anaphase II (Fig. 3E–F″), the intensity of H3S10-*P* staining on the maternal autosomes declined progressively starting from centromeric regions of the chromosomes to the more distal ones. Interestingly, the undivided X_m chromosome that remained attached to the polar complex, also showed a progressive reduction of H3S10-*P* signal towards the tip of the sister chromatids (Fig. 3E,E′). At the end of anaphase II, when one maternal group of chromatids reaches the undisjoined X_m chromosome to constitute the future sperm nucleus, no H3S10-*P* signals were detectable in the chromosomes. At this stage, the X_m chromosome sister chromatids can be discerned because appear separated along their length (insets in Fig. 3F′). Thus, at the conclusion of the second meiotic division, H3S10-*P* was only present in the bulk of paternal chromosomes eliminated at the end of meiosis I (Fig. 3G,G′, p); however, during spermatid differentiation, no staining was detected with antibody against H3S10-*P* (Fig. 3H).

Distribution of phosphorylated H3S28 in *S. ocellaris* spermatocytes during meiotic division

The immunolocalisation of H3S28-*P* in *S. ocellaris* meiotic divisions (Fig. 4) revealed a very similar pattern to that of histone H3S10-*P*. Intranuclear staining of prophase chromosomes was detected as chromosomes condense before they enter the ‘anaphase-like’ stage of first meiosis (Fig. 4A,A′). Similarly to H3S10-*P* staining, at meiosis I, only paternal chromosomes were recognised by the H3S28-*P* antibody. However, H3S28-*P* staining appeared less uniform and in a more speckled pattern than that of H3S10-*P* in the discarded paternal chromosomes. At the meiosis I–II transition (Fig. 4C,C′), when maternal chromosomes located at the polar complex re-condense, they become significantly H3S28 phosphorylated, except at one chromosome tip. As found for H3S10-*P* staining, during metaphase II it was evident that this chromosome corresponds to the X_m chromosome (Fig. 4D,D′). Similarly, during anaphase II progression (Fig. 4E–F′), a reduction of H3S28-*P* antibody staining was observed in the separated maternal chromatids, as well as along the X_m chromosome.

Distribution of phosphorylated H3T3 in *S. ocellaris* spermatocytes during meiotic division

In contrast to staining with antibodies against H3S10-*P* and H3S28-*P*, *S. ocellaris* condensing chromosomes at prophase were devoid of H3T3-*P* marks (Fig. 5A). H3T3-*P* signals were first detected at the ‘anaphase-like’ stage of meiosis I (Fig. 5B,B′) where, similarly to H3S10-*P* and H3S28-*P* staining, H3T3-*P* labelling was restricted to the highly condensed paternal set of chromosomes. Likewise,

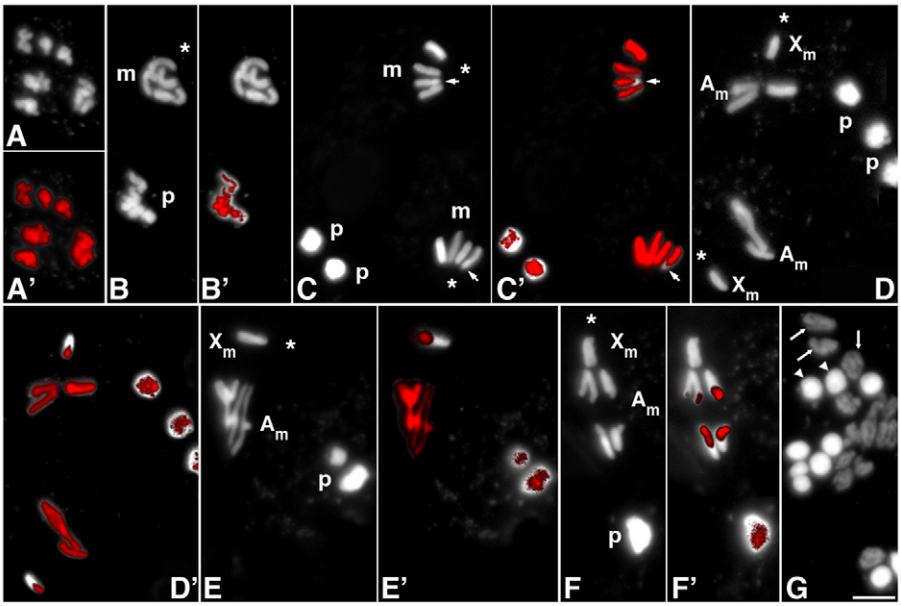

Fig. 4. Distribution of histone H3S28-*P* in *S. ocellaris* spermatocytes during meiotic divisions. (**A**–**G**) Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. (A′–F′) Indirect inmunolabelling with H3S28-*P* antibody and merged images where antibody staining is in red. (A,A′) Prophase nucleus showing antibody staining of condensed chromosomes. (B,B′) Anaphase-like stage showing maternal chromosomes devoid of antibody signals in contrast to paternal chromosomes. (C,C′) Two spermatocytes at the meiosis I–II transition; the condensed maternal chromosomes at the pole exhibit H3S28-*P* staining except at one chromosome end (arrows); paternal chromosomes show clustered H3S28-*P* signals, most probably corresponding to the centromeric regions. (D,D′) Metaphase II; the antibody decorates A_m chromosomes at the equatorial plate; X_m chromosome exhibits partial H3S28-*P* staining being the X_m chromosome centromeric end devoid of staining. Chromosomes from two cells are in this view, and therefore two individual X chromosomes are seen. (E,E′) Early anaphase II. (F,F′) Anaphase II showing the disappearance of H3S28-*P* signals at the X_m chromosome and labelling reduction to the A_m chromatid ends. (G) Spermiogenesis; spermatid nuclei (arrows) and eliminated chromatin (arrowheads) in buds lack antibody signals. Scale bar: 10 μm.

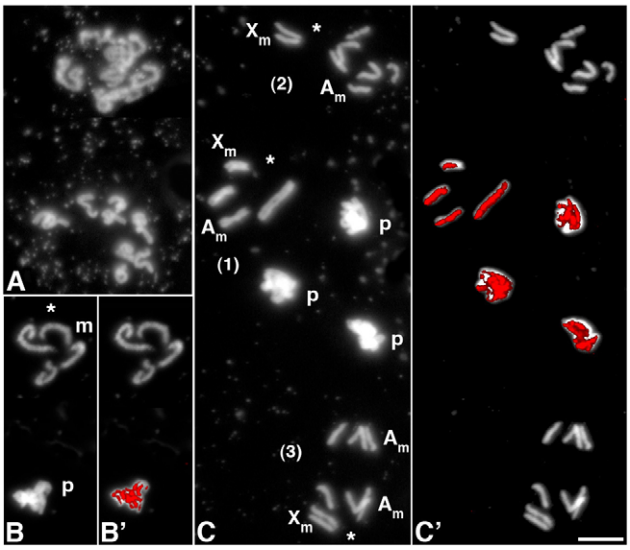

Fig. 5. Distribution of histone H3T3-*P* in *S. ocellaris* spermatocytes during meiotic divisions. (**A–C′**) Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. (B′–C′) Indirect immunolabelling with H3T3-*P* antibody and merged images where antibody staining is in red. (A′) Prophase nuclei undergoing chromosome condensation where H3T3-*P* signals are not detected. (B,B′) Late anaphase-like stage of meiosis I where maternal chromosomes appear less condensed than the paternal set; H3T3-*P* staining is restricted to paternal chromosomes. (C,C′) Three adjacent spermatocytes at meiosis II where one is at metaphase (1) and the other two at anaphase (2,3); at metaphase, H3T3-*P* antibody decorates the maternal chromosomes excluding the X_m chromosome centromeric end; at anaphase, all maternal chromosomes (X_m+A_m) are devoid of antibody staining, whereas the eliminated paternal chromosomes maintain H3T3-*P* signals. Scale bar: 10 μm.

H3T3-*P* antibody labelling was completely absent in the less condensed maternal set located at the polar complex of meiosis I. Moreover, at metaphase II, although the maternal chromosomes ware extensively decorated with H3T3-*P* antibody (Fig. 5C,C′), the non-disjoining X_m chromosome exhibited, again, lack of antibody staining at the centromeric end. Unlike the H3S10-*P* and H3S28-*P* staining, at the onset of anaphase, H3T3-*P* signals were dramatically reduced in all chromosomes, including the X_m chromosome (Fig. 5C,C′). From these results, we conclude that in addition to staining the paternal chromosomes in the late anaphase-like stage of meiosis I, phosphorylated histone H3T3 specifically associates to the metaphase stage of meiosis II and that the X_m chromosome at metaphase is H3T3 underphosphorylated at the centromeric end.

Distribution of phosphorylated H3T11 in *S. ocellaris* spermatocytes during meiotic divisions

The immunolocalisation of H3T11-*P* revealed that at prophase all chromosomes were decorated with H3T11-*P* antibody (arrow in Fig. 6A). At the anaphase-like stage of meiosis I, the antibody exclusively labelled maternal chromosomes and not the paternal set, as seen in all the spermatocytes of a cyst in Fig. 6A′. Thus, at meiosis I, the H3T11-*P* staining pattern was the reverse of that revealed by H3S10-*P*, H3S28-*P* and H3T3-*P* antibodies. This result was confirmed by double-immunofluorescence with H3T11-*P* and H3S10-*P* antibodies, where, as expected, both parental sets were stained (not shown). At the meiosis I–II transition (Fig. 6B,B′), maternal chromosomes remained labelled with H3T11-*P*, but the X_m chromosome was now unlabelled at the centromeric end, as confirmed next in observations of metaphase II (Fig. 6C,C′). Thus, these results strongly suggest that the X_m chromosome at the onset of meiosis II undergoes dephosphorylation of H3T11 at the centromeric end. Thus, the H3T11-*P* staining pattern at metaphase II (Fig. 6C′) is identical to that described above for the other histone H3 modifications. At anaphase II (Fig. 6D,D′), however, H3T11-*P* staining was detected along the chromosomes until the end of anaphase unlike the earlier loss of staining described above for the other antibodies.

Histone H3 phosphorylation in *S. coprophila* male meiotic divisions

We next investigated the distribution of four histone H3-*P* forms in *S. coprophila* spermatocytes undergoing meiotic division (Fig. 7). In addition to the ordinary chromosomal complement, *S. coprophila* poses germline L chromosomes that are paternally and maternally inherited (reviewed in Goday and Esteban, 2001; Greciano and Goday, 2006). In males, all L chromosomes segregate together with the maternal set at the first meiotic division. At the second meiotic division, L chromosomes undergo normal disjunction along with the maternal autosomes.

In meiotic prophase nuclei (Fig. 7A–D), immunostaining with H3S10-*P*, H3S28-*P*, H3T3-*P* and H3T11-*P* antibodies revealed that the ordinary chromosome complement of *S. coprophila* exhibits identical staining properties to those found in *S. ocellaris*. That is, all chromosomes appear phosphorylated for H3S10, H3S28 and H3T11, but not for H3T3. L chromosomes, instead, differ with respect to the ordinary chromosomes in that no labelling was detected with H3S10-*P* and H3S28-*P* antibodies (Fig. 7A,B). Because L chromosomes are highly heterochromatic, they display different cell cycle condensation timing. Consequently, the lack of H3S10-*P* and H3S28-*P* signals on the L chromosomes possibly reflects a lower condensation level of these chromosomes with respect to the rest, at this particular meiotic stage. However, in all our observations and as shown in Fig. 7D, ordinary chromosomes plus L chromosomes are entirely decorated with H3T11-*P* antibody.

During *S. coprophila* meiosis I and meiosis II, the four histone H3-*P* forms exhibited identical chromosomal distribution to that described for *S. ocellaris*. At meiosis I (Fig. 7E–H), maternal chromosomes plus L chromosomes were labelled only with H3T11-*P* antibody, whereas the discarding, more condensed, paternal set exhibited H3S10-*P*, H3S28-*P* and H3T11-*P* signals and lacked H3T11-*P* labelling. At metaphase II (Fig. 7I–L), the maternal autosomes plus the L chromosomes were decorated with the four antibody staining patterns, whereas the X_m chromosome, which remained attached to the first single pole was devoid of antibody staining at the centromeric end. At anaphase II, the four antibody staining patterns were identical to those found in *S. ocellaris* (not shown). From these results we conclude that in both *Sciara* species, the centromeric end of the un-disjoined X_m chromosome is specifically under-phosphorylated on histone H3.

Another intriguing observation referred to the staining behaviour of paternal chromosome, which, following meiosis I, remained congregated in the cytoplasmic bud of the spermatocyte. As mentioned above, paternal chromosomes in *S. ocellaris* usually move as a tight group into the bud, whereas in *S. coprophila*,

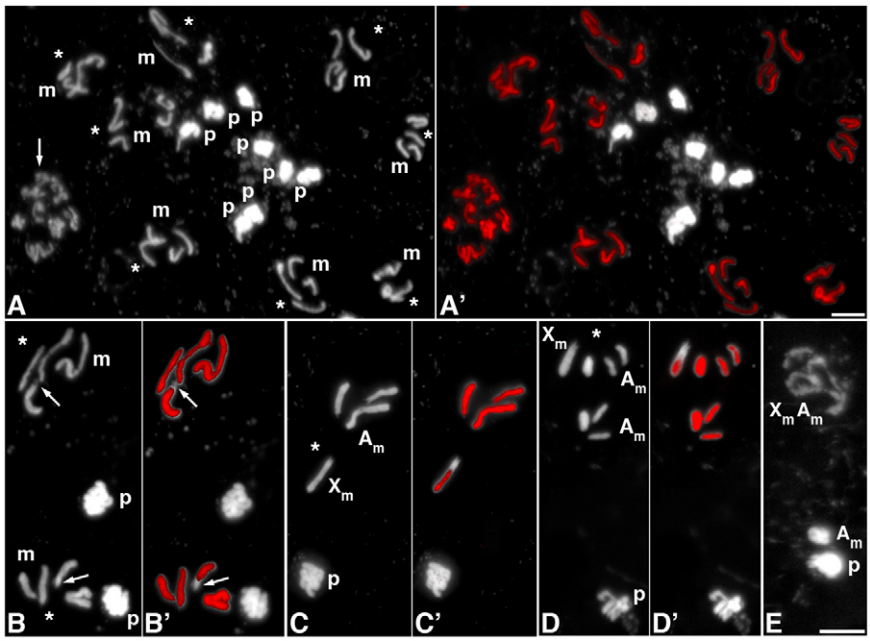

Fig. 6. Distribution of histone H3T11-*P* in *S. ocellaris* spermatocytes during meiotic divisions. (**A**–**D′**) Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. (A′,D′) Indirect immunolabelling with H3T11-*P* antibody and merged images where antibody staining is in red. (A,A′) A cyst with eight spermatocytes at meiosis I (anaphase-like stage) showing the typical radial arrangement where the discarding paternal chromosomes congregate in buds in the lumen; arrow in A denotes a prophase nucleus from an adjacent cyst, at an earlier stage, where condensed chromosomes are H3T11-*P* labelled (A′); H3T11-*P* antibody decorates only maternal chromosomes in all spermatocytes. (B,B′) Two cells at the meiosis I–II transition; maternal chromosomes (X_m+A_m) exhibit H3T11-*P* staining except at one end of the X_m chromosome (arrows). (C,C′) Metaphase II; the antibody decorates the maternal autosomes and the X_m chromosome excluding its centromeric end. (D,D′) Anaphase II showing that the two groups of maternal autosomes are entirely labelled except for the tip of the X_m. (**E**) Spermatocyte at the end of meiosis II; the future sperm-nucleus maternal components (X_m+A_m), the discarded maternal X-null chromatid set (A_m) and the paternal chromosomes (p), are all devoid of antibody staining. Scale bar: 10 µm.

individual paternal chromosomes can be often identified. In the present analysis, on several occasions we first detected in *S. ocellaris* spermatocytes, a small region protruding from the mass of paternal chromatin that was clearly devoid of H3S10-*P* and H3S28-*P* staining (arrows in Fig. 3A′,B′,E′). Moreover, in *S. coprophila*, we were able to discern that one of the acrocentric paternal chromosomes clearly lacked H3S10-*P* and H3S28-*P* labelling at one chromosome end (Fig. 7J,K). In view of this finding, we decided to investigate whether this particular paternal chromosome could correspond to the X chromosome. For this purpose, we performed in situ hybridisation with a rDNA probe known to localise in the short arm of the X chromosomes, next to the centromere (Crouse, 1977; Crouse et al., 1977). Fig. 8A,A′ shows a *S. coprophila* spermatocyte at metaphase II immunostained for H3S28-*P*. The four paternal chromosomes segregated into the bud during meiosis I can be clearly distinguished, including the centromeric region in three of them that are highly labelled by the antibody. The rDNA FISH analysis showed, as expected, rDNA sequences located at the centromeric end of the maternal X chromosome devoid of H3S28-*P* label (X_m in Fig. 8A,A′). Similarly, rDNA signals were detected at the distal part of the H3S28-*P*-unstained chromosomal region in one of the paternal chromosomes. These results led us to conclude that this chromosome with the unstained tip unequivocally corresponds to the paternal X chromosome (X_p in Fig. 8A′). Therefore, our data strongly suggest that at male meiosis, both parental X chromosomes are under-phosphorylated on histone H3 at the chromosomal end containing the centromere. Moreover, as expected, in other *Sciara* dividing tissues, histone H3-*P* patterns are identical between the X chromosomes and the rest of the chromosomes (an example of mitotic neuroblasts immunostained with H3S10-*P* antibody is shown in Fig. 9).

Distribution and timing of phosphorylated H3 forms during *Sciara* male meiosis

Based on all our results, in Table 1 we summarise the differential distribution of histone H3-*P* forms in *Sciara* ordinary chromosomes in relation to the main meiotic stages. At prophase, chromosome condensation correlated with an increase in the levels of H3 phosphorylation at Ser10, Ser28 and Thr11. At the anaphase-like stage, although the condensed paternal chromosomes remained highly phosphorylated, maternal chromosomes decondensed to some extent and became dephosphorylated at histone H3 Ser10 and Ser28 but remain phosphorylated at Thr11. As maternal chromosomes initiate recondensation (meiosis I–II transition) to enter metaphase II, significant phosphorylation at Ser10 and Ser28 occurred again, and in addition, for the first time at H3T3. Thus, the four H3-*P* modifications spread over the metaphase chromosomes are also found in the X chromosome, but not at the centromeric end. Interestingly, phosphorylation at H3T3 is the only H3 form that is restricted to the metaphase stage.

Discussion

An intriguing finding of this work is that at the onset of the second meiotic division, the maternal X chromosome undergoes normal H3 phosphorylation except at the centromeric end, which is under-phosphorylated for the four H3-*P* forms. We provide here the first evidence linking the under-phosphorylated H3 status of the X

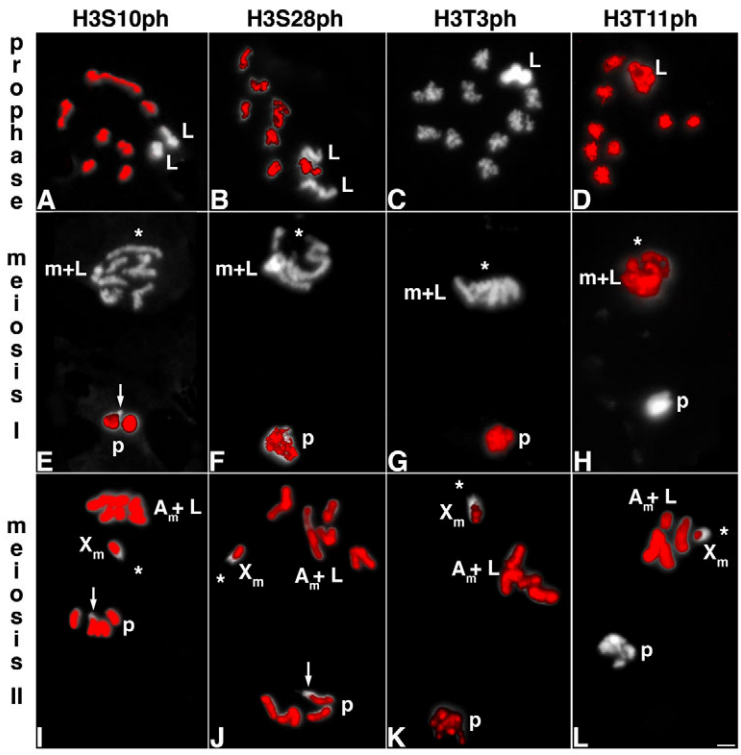

Fig. 7. Distribution of histone H3S10-*P*, H3S28-*P*, H3T3-*P* and H3T11-*P* in *S. coprophila* spermatocytes undergoing meiotic divisions. Chromosome DAPI staining; m and p refer to the maternal and paternal chromosome complement, respectively; asterisk indicates the position of the single spindle pole. Indirect immunolabelling with antibodies indicated in the top and merged images where antibody staining is in red. (**A**–**D**) Prophase; H3S10-*P*/H3S28-*P* staining is identical; both antibodies stain all chromosomes except the L chromosomes (A,B); H3T3-*P* signals are not detected in the chromosomes (C) whereas H3T11phos antibody decorates all chromosomes (including L chromosomes) (D). (**E**–**H**) Anaphase-like stage; antibodies against H3S10-*P*, H3S28-*P* and H3T3-*P* decorate only paternal chromosomes, whereas maternal chromosomes and L chromosomes, at the polar complex, are devoid of staining (E–G); arrow in E denotes a small region on the eliminated paternal chromosomes devoid of H3S10-*P* labelling; H3T11-*P* antibody decorates maternal chromosomes and L chromosomes whereas paternal chromosomes lack antibody staining (H). (**I**–**L**) Metaphase II; X_m chromosome exhibits lack of staining at the centromeric end with all the antibodies; maternal and L chromosomes at the metaphase plate are stained by all the antibodies; whereas antibodies against H3S10-*P*, H3S28-*P* and H3T3-*P* stain paternal chromosomes, no staining is detected with H3T11-*P* antibody; arrows in I,J denote the lack of antibody staining at a chromosomal end in the paternal set. Scale bar: 10 μm.

chromosome centromeric region with meiotic non-disjunction of this chromosome in sciarid flies.

Histone H3 phosphorylation, chromosome condensation and paternal chromosome elimination at meiosis I

Many studies indicate that histone H3 phosphorylation is required for chromatin condensation before chromosome segregation at both mitosis and meiosis (Gurley et al., 1978; Van Hooser et al., 1998; Goto et al., 1999; Wei et al., 1999; Giet and Glover, 2001). Accordingly, we found significant levels of H3S10-*P*, H3S28-*P* and H3T11-*P* in *Sciara* spermatocytes undergoing chromosome condensation at prophase. Moreover, our results also confirmed that the timing of H3S10 and H3S28 phosphorylation (and dephosphorylation) is very similar, as previously reported in other dividing cell types (Nowak and Corces, 2004; Xu et al., 2009). By contrast, H3T11-*P* does not always overlap with phosphorylation of H3S10 and H3S28, given that the H3T11-*P* modification was also found in less condensed chromosomes that were under-phosphorylated at H3S10 and H3S28 (see Table 1 and L chromosomes in Fig. 7). However, H3T3-*P* specifically associates with chromosomes displaying the highest degree of compaction during the meiotic process (metaphase II).

A detailed description of the cellular mechanisms involved in the highly atypical separation of the two parental sets during male meiosis I was given in previous studies (Gerbi, 1986; Goday and Esteban, 2001). An important feature is that, before the anaphase movements of meiosis I, each parental set of chromosomes in the prophase nuclei occupies distinct compartments, with the maternal set always closest to the polar complex of the monopolar spindle (Kubai, 1982; Kubai, 1987). In view of this observation, it was concluded that the first meiotic spindle maintains a pre-existing segregation of the two chromosome sets while the distance between them increases (Kubai, 1982). The proximity of maternal chromatin to a single giant pole generating numerous microtubules ensures that, following breakdown of the nuclear membrane, the maternal chromosomes are retained at the polar complex during meiosis I (Gerbi, 1986; Goday and Esteban, 2001). When covalent histone modifications were analysed during germline development in *S. ocellaris*, differences in the acetylation or methylation of histones H3 and H4 were found between maternal and parental chromosomes (Goday and Ruiz, 2002; Greciano and Goday, 2006). Concerning male meiosis I, whereas maternal chromosomes at the polar complex are acetylated at histones H3 and H4, the eliminating paternal chromosomes are, instead, under-acetylated and methylated at histones H3 and H4 (Goday and Ruiz, 2002; Greciano and Goday, 2006). Here, we show that phosphorylation of histone H3 differs between the two separating parental sets of chromosomes at male meiosis I, and that such differences in H3 phosphorylation coincide with the extent of chromosome condensation. Thus, the unravelling of maternal chromosomes (at the polar complex) correlates temporarily with dephosphorylation at H3S10 and H3S28, whereas the condensed paternal set (segregating to the bud) remains phosphorylated at H3S10 and H3S28. This finding is in good agreement with previous data, where *Sciara* paternal chromosomes at meiosis I were found to exhibit significant enrichment in H4K20 methylation (Greciano and Goday, 2006), a histone modification associated with densely packed chromatin (Rice et al., 2002). The present data on histone H3 phosphorylation give further support to our previous model that relates intranuclear chromosome arrangements, histone covalent modifications and chromosome elimination in *Sciara* germline nuclei (Greciano and Goday, 2006).

Histone H3 phosphorylation and X_m chromosome non-disjunction at meiosis II

From our analysis, the *Sciara* male meiosis I–II transition is marked by the presence of the four H3-*P* forms, coincident with a further compaction of maternal chromosomes before metaphase II entry

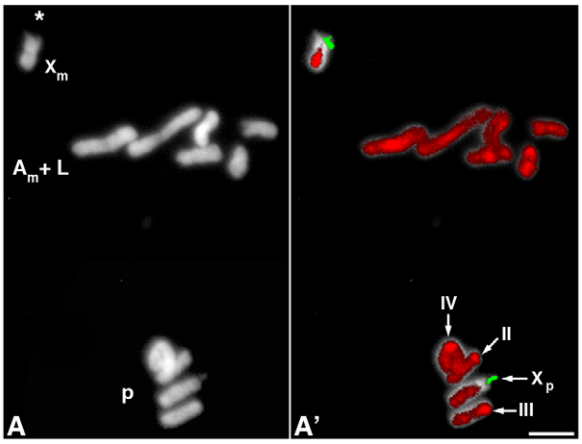

Fig. 8. Distribution of rDNA in a *S. coprophila* spermatocyte at methaphase II. (**A**) Chromosome DAPI staining. (**A′**) rDNA in situ hybridisation (green) and H3S28-*P* immunostaining (red). rDNA sequences locate to the centromeric regions of X_m and X_p chromosomes; both X chromosomes are devoid of H3S28-*P* signals at the rDNA-chromosomal tip (see arrows in A′); note that the centromeric regions of the rest of the chromosomes are strongly labelled by the antibody; arrows indicate the centromere position in the paternal chromosomes (chromosome II, III and IV). Scale bar: 10 µm.

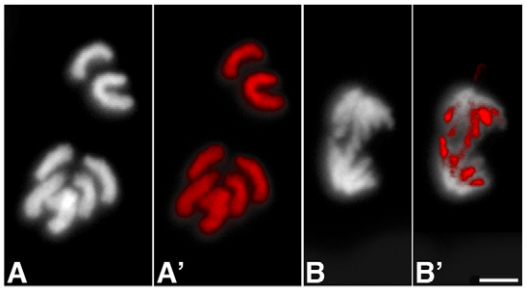

Fig. 9. Distribution of phosphorylated histone H3 at Ser10 in mitotic chromosomes from *S. ocellaris* neuroblasts. Metaphase (**A**) and anaphase (**B**) chromosome DAPI staining. (**A′**,**B′**) Indirect immunolabelling with H3S10-*P* antibody and merged images where antibody staining is in red. All chromosomes at metaphase are similarly stained by the antibody (A′); middle-anaphase showing a reduction of the antibody staining to the chromosome distal regions. Scale bar: 10 µm.

(see Table 1). Therefore, H3 phosphorylation of maternal chromosomes congregated at the polar complex site precedes the migration of the autosomes to align in a metaphase II plate. A particularly good example is seen in Fig. 4C′ where *S. ocellaris* maternal chromosomes stained with the anti-H3S28-*P* antibody line up with the centromeres oriented towards the single pole in the primary spermatocyte. Taking into consideration all our observations, we conclude that it is at this particular 'pre-metaphase stage' when local deficiencies in H3 phosphorylation are established at the centromeric end of the X_m chromosome. In view of our results, it seems very reasonable to question whether such H3 phosphorylation modifications are responsible for the persistent association of X_m chromosome with the polar complex microtubules throughout meiosis II. That is, if the X_m chromosome failure to align on the metaphase II plate and, consequently, to undergo chromatid disjunction depends on the deficiency in H3 phosphorylation at the centromeric region. We believe that this is so, and we discuss this further below in light of recent data that demonstrate the crucial role of histone H3 phosphorylation in centromere function during mammalian cell division (Kelly et al., 2010; Wang et al., 2010).

We have demonstrated that during anaphase II histone H3 dephosphorylation at Ser10 and Ser28 begins at the centromeric regions of the chromosomes and extends along the chromosome arms. This is consistent with observations of the distribution of H3S10-*P* in anaphase chromosomes from *Drosophila* syncytial mitosis, where dephosphorylation events originate at the centromere region (Su et al., 1998). In this respect, similar observations were obtained in early *Sciara* embryonic somatic divisions (not shown), as well as in neuroblast cells (Fig. 9), suggesting that a gradual H3S10 and H3S28 dephosphorylation pattern might be a general occurrence. Consistently, the un-disjoined X_m chromosome lacking H3-*P* at the centromeric end, also exhibits non-uniform dephosphorylation of H3S10 and H3S28 that, in this chromosome, initiates at the proximal region of chromosome arms. So it seems that, at least in the case of the X_m chromosome, phosphorylation or dephosphorylation of histone H3 does not necessarily begin at the centromere site to extend then along the chromosome arms. However, from our observations in *Sciara* spermatocytes at anaphase II, a gradual loss of H3T3 and H3T11 phosphorylation along the chromosome arms could not be discerned. It appears that histone H3T3 phosphorylation is dramatically reduced at the onset of anaphase in all chromosomes, whereas phosphorylated H3T11 persists until very late anaphase (see Table 1). Taken as a whole, the results support differential phosphorylation and

Table 1. Summary of histone H3 phosphorylation distribution during the first and second male meiotic divisions in *Sciara*

	Meiosis I							Meiosis II					
	Prophase		Anaphase-like			Meiosis I–II transition		Metaphase		Early anaphase		Late anaphase	
	m	p	m	p									
	A_m,X_m	A_p,X_p	A_m,X_m	A_p	X_p	A_m	X_m	A_m	X_m	A_m	X_m	A_m	X_m
H3S10-*P*	+	+	–	+	+/–	+	+/–	+	+/–	+	+/–	–	–
H3S28-*P*	+	+	–	+	+/–	+	+/–	+	+/–	+	+/–	–	–
H3T3-*P*	–	–	–	+	+/–	+	+/–	+	+/–	–	–	–	–
H3T11-*P*	+	+	+	–	–	+	+/–	+	+/–	+	+/–	+	+/–

m, maternal chromosomes; p, paternal chromosomes; A_m, maternal autosomes; X_m, maternal X chromosome; A_p, paternal autosomes; X_p, paternal X chromosome; +/– denotes antibody staining for a large part of the chromosome except at the centromeric end. Paternal chromosomes are only shown in meiosis I when they are eliminated. L chromosomes (not included in table) behave identically to maternal chromosomes from the initiation of the first meiotic segregation process.

dephosphorylation timing between histone H3-*P* forms along the meiotic process in *Sciara*. Importantly, all the H3-*P* forms first arise at the meiosis I–II transition, and remain until the onset of anaphase II.

Histone H3 phosphorylation and X_m centromere inactivation

The kinase Aurora B phosphorylates H3S10 and H3S28 and both H3-*P* modifications are implicated in chromatin compaction, which in turn, is required for chromosome congression and proper chromosome segregation during mitosis and meiosis (Gurley et al., 1978; Wei et al., 1998; Wei et al., 1999; Goto et al., 1999; Hsu et al., 2000; Giet and Glover, 2001) (reviewed by Nowak and Corces, 2004). Aurora B is a component of the CPC (chromosomal passenger complex) that also contains INCENP, Survivin and Borealin/Dasra (Giet and Glover, 2001; Ruchaud et al., 2007). At the beginning of the M phase, the CPC associates with condensing chromatin, accumulates at the inner centromere at metaphase and relocates onto the central spindle at anaphase (Giet and Glover, 2001; Carmena and Earnshaw, 2003). Importantly, the CPC controls chromatin-dependent spindle assembly and processes at the centromere (Ruchaud et al., 2007). Depletion of Aurora B kinase in *Drosophila* S2 cells greatly reduces the levels of H3S10 phosphorylation and produces important alterations in the condensation of mitotic chromosome (Giet and Glover, 2001). One of the roles of Aurora B, moreover, is to ensure chromosome bi-orientation at metaphase and to correct mono-oriented attachments to the spindle (Shannon and Salmon, 2002). Taking this information into consideration and given that at metaphase II, the mono-oriented X_m chromosome is clearly under-phosphorylated for H3S10 and H3S28 at the centromere region, it seems reasonable to assume that this region in the X_m chromosome is deficient for Aurora B activity and that it is most probably less condensed than a regular centromeric region.

Phosphorylation of H3T3 is dependent on Haspin, a conserved kinase that functions in mitosis and has homologues in all the main phyla, including fungi, animals and plants (Dai et al., 2005; Higgins, 2010). Haspin, localises predominantly to chromosomes, and phosphorylates histone H3T3 during mitosis at the chromosome arms and at the inner centromeres between the regions delineated by the centromere-specific histone CENP-A (Polioudaki et al., 2004; Dai et al., 2005; Dai et al., 2006; Higgins, 2010). Interestingly, Haspin RNAi in mammalian cells causes partial metaphase figures with numerous misaligned chromosomes, where many of them become trapped near the spindle poles (Dai et al., 2005). Such chromosomes, as demonstrated using anti-centromere antibodies, are constituted by mono-oriented sister chromatid pairs (Dai et al., 2005). It is noteworthy that the *Sciara* X_m chromosome at metaphase II displays an identical cytological phenotype to that described for misaligned chromosomes in Haspin-depleted cells. Considering that the centromeric region of X_m chromosome lacks H3T3-*P*, it is possible to assume that, similarly to Aurora B, the centromere of the X_m chromosome is deficient in Haspin kinase activity. This could explain the misalignment of X_m chromosome at metaphase II and its permanent attachment to the spindle pole until the end of anaphase II.

Recently, the essential functional role of Haspin during mitotic division has been further investigated in mammalian cells (Kelly et al., 2010; Wang et al., 2010). From such studies, it emerged that Haspin is required for the accumulation of CPC at the centromeres, and that the CPC subunit Survivin binds directly to phosphorylated H3T3 (Wang et al., 2010). Most important, H3T3-*P* is recognised by an evolutionarily conserved binding pocket in the BIR domain of Survivin (Kelly et al., 2010). As concluded, H3T3-*P* positions the CPC to the centromeres to regulate Aurora-B-selected targets and the interaction between H3T3-*P* and CPC is mediated by Survivin (Kelly et al., 2010; Wang et al., 2010). Taking into consideration these data and our results, it is highly predictable that the mere lack of H3T3-*P* in the centromeric region of the X_m chromosome (at the meiosis I–II transition) prevents the CPC recruitment and thus leads to a non-functional centromere.

The *CE* and histone H3 phosphorylation in the X chromosome

Early work in sciarid flies identified a *cis*-acting locus, the *CE*, which regulates X-centromere activity during *S. coprophila* male meiosis and is contained in the heterochromatin proximal to the X centromere (Crouse, 1960; Crouse, 1977; Crouse, 1979; Gerbi, 1986). Although the molecular nature of the *CE* is still unknown, it has been considered that it is capable of modifying normal centromere function of the X chromosome during male meiosis II (Gerbi, 1986). The present results support our view that the *CE* modifies the X_m centromeric function, causing X_m chromosome non-disjunction, by inhibiting global H3 phosphorylation at the centromeric chromatin. Therefore, it seems that the *CE* governs the inability of the chromatin at this particular X chromosome region to become H3 phosphorylated, unlike the rest of the chromosome. Consistently, we found that H3 phosphorylation was also inhibited at the centromeric region of the paternal X chromosome, which together with the paternal autosomes, is eliminated at meiosis I in both *Sciara* species. In this regard, the deficiency in H3 phosphorylation at the X_p centromeric region does not affect the X_p chromosome segregation modality at meiosis I, in either *S. ocellaris* or *S. coprophila*. This interesting observation gives further support to the conclusion that the presence of organised kinetochores in paternal chromosomes seems not to be necessary for their regular elimination at meiosis I (Goday and Esteban, 2001).

An understanding of the organisation of the heterochromatic sequences conforming the *CE* and the mechanisms established to inhibit chromatin H3 phosphorylation in *cis* are important questions that remain to be answered.

Materials and Methods

Fly culture

S. ocellaris and *S. coprophila* were raised at 20°C as described elsewhere (Rieffel and Crouse, 1966).

Fixation

Sciara prepupae were dissected in 15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.5 mM espermidine, 0.1% Triton X-100 solution to remove the testes. For each experiment with a different H3-*P* antibody at least ten testes were processed. The testes were immediately fixed with 1% paraformaldehyde, 0.1% Triton X-100 in PBS for 3 minutes. They were then incubated in 50% acetic acid, 1% paraformaldehyde for 1 minute and squashed. Slides were frozen in liquid N_2 to remove coverslips, postfixed in 3.7% formaldehyde for 10 minutes and extensively washed in PBS. For mitotic neuroblasts analysis brains were removed from third instar larvae and fixed as above.

Immunostaining and microscopy

All slides were washed in PBS (3×10 minutes), incubated in PBS containing 1% Triton X-100 (10 minutes) and in PBS with 3% BSA and 0.1% Tween for 1 hour at room temperature. The primary antibodies were rabbit polyclonal anti-H3S10-*P*, anti-H3S28-*P*, anti-H3T3-*P*, anti-H3T11-*P* (Upstate Biotechnology) diluted 1:40 to 1:100; mouse monoclonal anti-β-tubulin (Amersham) diluted 1:200. Secondary antibodies were FITC- and Cy3-conjugated anti-rabbit (Southern Biotechnology) and Rhd-conjugated anti-mouse Ig (Dakoppats). Secondary antibodies were diluted 1:50 for FITC-conjugated antibody and Rhd-conjugated anti-mouse and 1:800 for

CY3-conjugated antibody. Primary antibody incubation was at 4°C overnight. Secondary antibody incubation was at room temperature for at least 1 hour. DNA was visualised with 4′6-diamino-2-phenylindole (DAPI) staining (0.1 mg/ml; 3 minutes) and preparations mounted in anti-fading solution. Observations were made under epifluorescence optics with a Zeiss axiophot microscope equipped with a Leica CCD camera. Digital images were processed using the AdobePhotoshop PS software.

In situ hybridisation

For fluorescence in situ hybridisation (FISH), the probe pDm238 (Tautz et al., 1988) containing a complete rDNA cistron of *Drosophila melanogaster* was used. The probe was labelled with digoxigenin using the DIG-Nick Translation Mix (Roche Diagnostic), according to the manufacturer's instructions. Slides previously incubated with H3-*P* antibodies were then processed for in situ hybridisation as described elsewhere (Goday et al., 2006).

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3

Caracterización de secuencias teloméricas de *S. coprophila*

3. Caracterización de secuencias teloméricas de *S. coprophila*

Para aislar y caracterizar las secuencias teloméricas se microdiseccionó la región telomérica 1A del cromosoma IV a partir de cromosomas politénicos (ver Figura 1). Los fragmentos de DNA diseccionados se amplificaron mediante DOP-PCR (Telenius *et al.*, 1992) y el DNA amplificado se clonó en el vector TOPO TA (Invitrogen). La genoteca generada contiene 300 clones, de los cuales se secuenciaron un total de 249. En contraste con el resultado obtenido del análisis descrito anteriormente para la heterocromatina centromérica del cromosoma X, en este caso, el análisis mediante *"BLAST" (Basic Local Alignment Search Tool)* de las secuencias teloméricas aisladas, reveló fragmentos de genes de la región telomérica 1A (Figura 2). Una vez descartados los clones derivados de genes y los correspondientes a amplificaciones no específicas, se procedió a analizar 109 clones mediante experimentos de hibridación *in situ* sobre cromosomas politénicos. Como resultado de éste análisis se observó que la señal de hibridación del clon 1Tel.29 en el cromosoma IV es intensa en la región telomérica 1A y débil en el otro telómero (Figura 3B). Por otra parte, la secuencia del clon 1Tel.29 indica que el fragmento deriva de una zona con repeticiones en tándem, siendo la unidad de repetición de 176 pb (negrita en la Figura 3A). Es importante destacar aquí, que se trata de la primera secuencia telomérica descrita en *S. coprophila*, y también que su grado de complejidad es similar al de las secuencias teloméricas descritas en otros dípteros, como es el caso de *Chironomus* (Martinez *et al.*, 2001; Diez *et al.*, 2006). La hibridación *in situ* sobre cromosomas politénicos y cromosomas mitóticos (Figura 3B y 4) reveló que dicha secuencia deriva de la región telomérica 1A, lo cual sugiere la presencia de secuencias de DNA con cierta homología en el otro telómero.

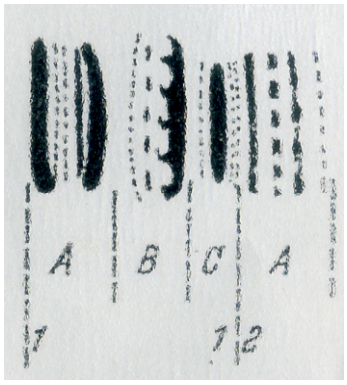

Figura 1. Extremo telomérico del cromosoma IV de *S. coprophila*. Diagrama mostrando la región telomérica 1 del cromosoma politénico IV según (Gabrusewycz-Garcia, 1964).

4TEL.01.C3 Putative E3 ubiquitin-protein ligase HERC2
AGCGGATGAAGAGTTACCCTGGAAATGAAATAATTGAAAATCTTCAGAAGCTACAGGGAAGAGTGTCTGTCAGAACGAAAC
TGCGTTCTGGTCAAAATGTCCTTTGACTATGATTGAATTAGGTCGACCGAGCTTCAGTCACAACTTTCATCTCTCATGAGT
CACACTTTGAAACGCAGAACTTAAAACTCACCTGAGAATTCGTCACCAATTCCATTTCACTCAGCTGTCCCGTCCAACTGA
ATTGTTGCGGAAAGTCTACCGTAACATCTCCGTTGTCGTTGATAGCCGTAACGACACCCACACTTCCTCGCACAACCGAA

4TEL.01.C7 tankyrase
GTGTCTGTCGTTCTTGATTCTTCATGTAGGCATCTGGTCAGGGAAAGTGTTTCGATTAAATTGACGTTCCGTTTCACATTT
AACGTTACAATCATACCTGCTCCATGAGCCAACAGCAGCGCACACAATTGTGTACGACCTTGGGCCGCTTCATGTAACGGT
GTAAATCCCCATCGATCGGTGGCATTCACAATCGTGTTGTGTTTGATCAATAGAGCAGCAATATCTAAATGGCCGTACGAT
GAGGCGTTATGAAGTGGTATCAGTCCACCCTTGTCCTGTGAATTAACTTCGGCGCCGTTCTCCAACAAAAACTCAGCCACT
TCGAAGTTATTGTAACCAGCAGCCAGATGAAGGGGTGTACTGTTCCGACCTACGAATTATTTCGTCCGGTTAAGTATCAAC
TCACGGCCACAACAACAA

4TEL.01.G10 Cytochrome P450-6a9
AGTGCGTGTTGGAGATGAAAGATATTCTGGCCCGATATACCACTGATGTTATCGGGAAATGTGCCTTCGGCATTGAGTGTA
ATAGGTAAGTAGTTGCCAGCGAGTCAAGTGTTGATATTGTCGGACTGAAGTTCGAGTATCGTTGTGAACTAATTCTGATGG
CTTGGTATTCTGCTCGGAATCTAATTTTTTCTTGTATTTCCAACGTTCTCGTCGTTACCAGTCTAACCAATCCCGATGCAG
AATTTTGGCGTATGGGAAAGCGAACCTTCGAAGCGCCAAGACACAAACCAATTATCACATTTTTGATCGGCTCATTTAAGC
GGCTCGCTGTGATGATACGAACAAAAGTTATACGGGACGATGTGAGTGACTTCTTTCTGAACATCGTTCACGAGACAGTCC
ATCATCGGAAAGCCAACGACATCCGAAGGAATGACTTCATGGACATTTTGATTAACTTGACCAATCAGGAAGAGGGATCGC
TAACCATCGATGAAATAGCTGCACAAGCGTTCGTGTTTTTCCTGG

4TEL.02.G5 Putative E3 ubiquitin-protein ligase HERC2
AGCCGAAGCAAGTTTAATGAGGCCACTGCAATGCACTCAGATTCTTGGTTAGGAGGATGTCTGATCTCACTGGTAATGGCA
CACACAATCGCTAAAAGTTGATCGATTAACCGGAACGTTGATTCGGACAGGTCGACAATGAACGGAAGTTTCTTAGCGATT
CCTGACGTGGACGTATTGCTCCATAGAATGGATTGTGCTGGACCACATGGTATTCCGGTTGTTCGTAGCGATGGAGGCGTA
ACTTGAATGGGTGTTGTAATCATCGTAGACGAGGATACCGACTTGGGACAAATCTGATTGTAATCATTGCGCCCAAAACCG
TAAACTTCACCATTACTCGTCATGAGCAGACAATGTGCGCTACCGACTGCCACATTGACAATATTTTTACCTTCCAATGAT
TCAATTCGTCGTGGATGCGGCAGGTAGTCGCACAACTCAGCATTCGCTTTCGATTTTTCATCGGACATATCAGCCGAACAA
GGTCGTGCACCGTTTTTGTTGCTCCACACGTACACCGATCCGTCAAAAGACAAAGCCACATTTTCA

4TEL.02.F6 Putative E3 ubiquitin-protein ligase HERC2
CCACACATGGTGAGTTAACTGGCGACTTGACCGTGGAAGAGATTGGAACGAAATTTCCGTTTCAGCTTATTACTCATCGCG
TCGACTCACTGTTAACCGGATTGAGTGAAGCACGATTAACGGATCCATTGGTATCGACGTGGTACACCATTGCGGACCGAT
TTTGCAAGGAGAACAGTTTCATCTGGCACCAAGACTTTC

4TEL.02.E7 Toll-related protein
ACTGAACTCACTGGAAGAACTTTGGCTGAACTACAACAAATTGAAAGTGATTTCGGCGGATTCGTTCAAGAACTTAAAGAG
CCTCAGGCTATTGCAGCTCAGCTTCAACAACATTGAGTCGGTTAATTTTACCTTAACCCCTACGACAGCAGGATCTCTTCC
TAATTTGACCAGCTTGAACCTTCTATTCAACAGCATTACCGAAATTCCAGACGGTACATTCACATTGCTCACCAATCTTCG
GGACTTGAATTTGTGTCGAAATCGAATCGAATTCCTGAGCTCGAGTTAAGCCACATCCGCACCTCGAGTCGGA

4TEL.02.D8 Chaperonin
CAGGTATTGCTGTCCGCATCGGAAGCAGCCGAAATGATATTGCGGGTGGACAATATCATTCGATGTGCGTCACGCAAGCGT
GTGCAAGATCGAGGAATGTGTTAGGTTCGGTTTTTCATTTTCGATTTTCGCGGAAAATGCTTTCGTTTTCACTTAACTTGT
CGCTTTAACTAAC

4TEL.02.C9 kinesin-like protein
AACACGAGCACGCGAAGCGTTCGGATGAGCAAGCGATCAAAGTGACATCAATGACGGACGAACAGAAAAAGGATTTCGCTG
CGACGGGAAATGATATTTCGAAAACGATGGCATCGATCACCAATCACACCAAACTGTTTGCGGCAGCTATGAAATCGTCGA
AGGTGAAAATCATCGAGCAGTTTGATAAGCAGAAAGGCATGGCAGCGGCAGCCTTAGCTAACATCGATAGCAAGATCTCGG
ATGGCATCGAGAAAATGAAGAGTACGACGCAGGATATCAGCGGCATCGTCAACGATGCTGACCAAAATCTTAAGATTGATT
CACAGGCCAACTTAAACTTCAAGCAAACTGTGACGACGTTCGTCAATTCCTTCGGCGCAGCGTCAAAGAAGAAGTTTGACA
ATTTGCGGACCATTGTCCACAATTTCCACGGCAATGACTTGAAGGTCTATTCGTCGAGCGGTAAGTGTTTCGCATTTGAAT
CTTTATCGACCGACATGTAACGTAACGACTCAATCTATCCATTCAGGCGACACGCCCGCTAGACGCGATTTCAACTATCCG
AAAGTACTGGCGGCAACATCGCCACATGATCGCATCGTTCGACGATTCTGGTCGACTCATAATGAATCGGAACTGGACGTA
TCGATGTCGATATCAGAGGTACAGACGGCGCAAAACAATCGATCTG

4TEL.02.F9 Mismatch repair ATPase
TGTAGACTGTTCATGGATTCGGCCTAACTTTCTACTTATTTTTTTCCTTAAGGTGTCAAGTTCGACAGTCCGGCAAAGCAG
AAAACGTTCACCGATAACTTGACCACGAACCAACTGGAATTTTTGCTCCAATCTCAAGGCATATTCTGCTACAAATTCGCT
CAATGCAACGAACTAAACGAATCGTTCAGAGAATTAAATGTTCGAGCCCGATGTCAGGAGGAACAAATTCTAAAAGAATTT
CTCTCTTGGTACACCCCTAACGTTCCGACCATCCAATACAACACCGACTTATACGCCAAATTGGACTGCATGATGGTCTTC
GCCGAAATTGCTACCAAATATGAACTAGTCCGTCCCGAATTGACGCACGAGAAAGCACTGGAGATCCGTCAAGGGCGCCAT
TTTCTGTTCAATTCAAAGA

Figura 2. Fragmentos de genes de la región 1A del cromosoma IV de *S. coprophila.*

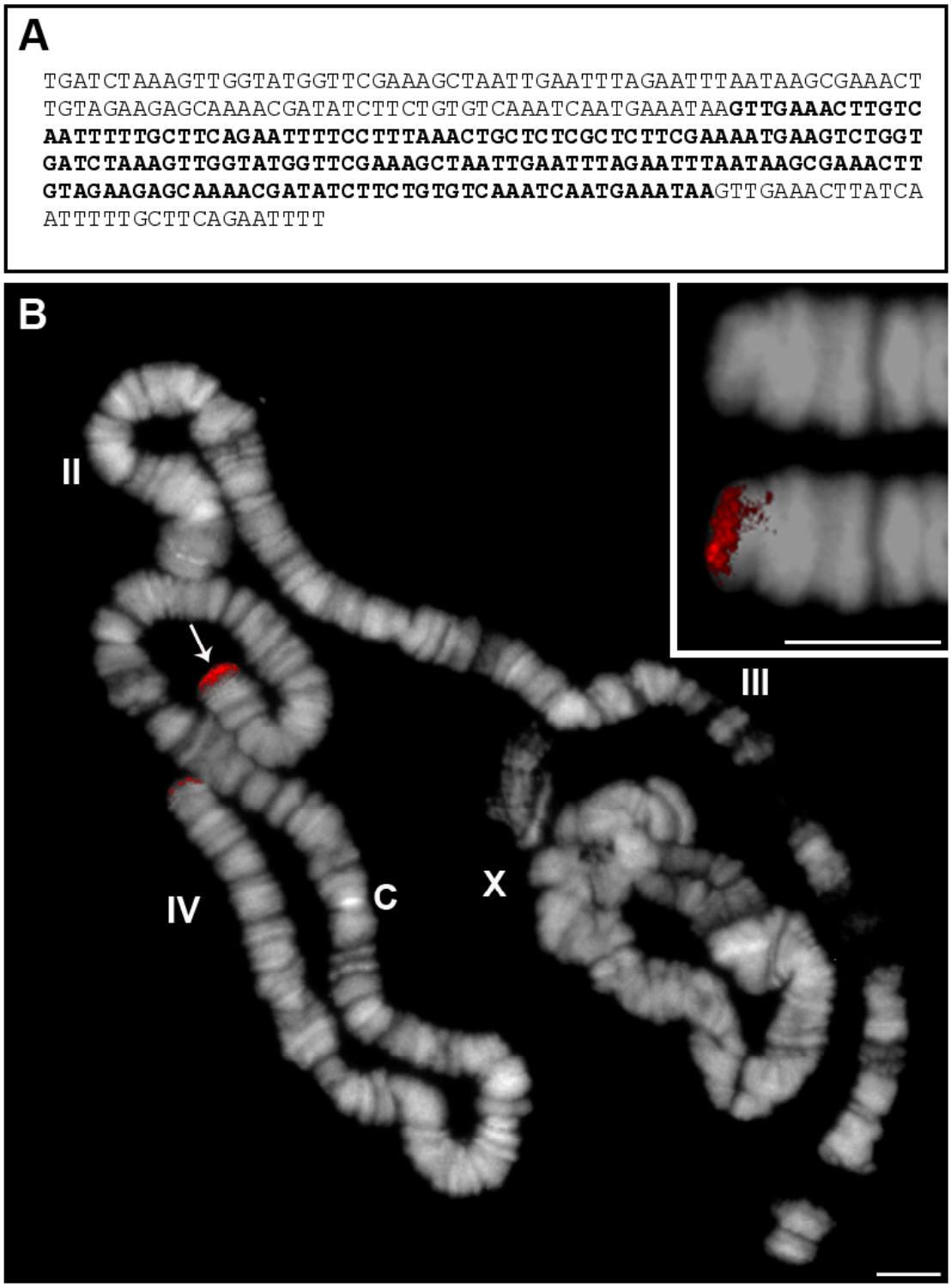

Figura 3. Hibridación *in situ* del clon 1Tel.29 en cromosomas politénicos de *S. coprophila*. (A) Secuencia del clon 1Tel.29. La unidad de repetición se indica en negrita. (B) Los cromosomas politénicos (**I**, **II**, **III** y **IV**) de las glándulas salivales de *S. coprophila* se han teñido con DAPI**.** La señal obtenida (rojo) se localiza en ambos telómeros del cromosoma IV, aunque la señal correspondiente a la región 1A (**flecha**) presenta una mayor intensidad. También se señala la posición del centrómero (**C**) del cromosoma IV que es metacéntrico. El inserto muestra una ampliación de la región terminal 1 del cromosoma IV. Barra, 10 μm.

Esto podría ser consecuencia del intercambio de secuencias a nivel de telómero debido a su proximidad intranuclear por la disposición de los cromosomas en la configuración Rabl (Horowitz *et al.*, 1984) al tratarse de cromosomas metacéntricos. Por último, es de interés mencionar que la existencia de secuencias específicas de un solo telómero ya ha sido descrito en otros Dípteros, como *Anopheles gambie*, donde en el cromosoma 2 el satélite 2L es específico del telómero de 2L (Biessmann *et al.*, 1996).

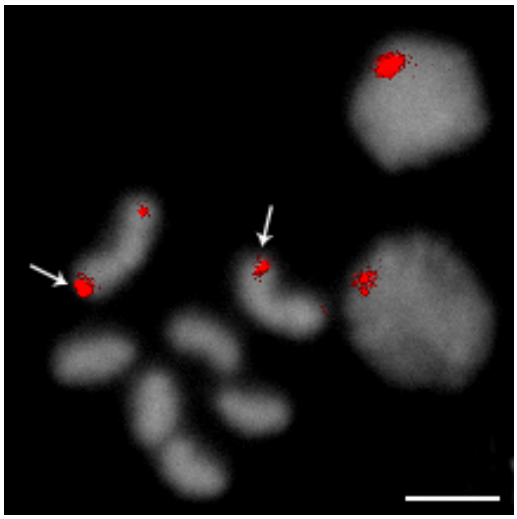

Figura 4. Hibridación *in situ* del clon 1Tel.29 en cromosomas mitóticos y núcleos interfásicos de neuroblastos de *S. coprophila*. Los neuroblastos se han teñido con DAPI. La señal obtenida (rojo) se localiza en los dos telómeros de los cromosomas IV, aunque se observa mayor intensidad en la región telomérica 1A (**flechas**). Barra, 10 μm.

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Conclusiones

CONCLUSIONES

1. Se ha microdiseccionado y microclonado la región centromérica del cromosoma X de *S. coprophila*. Las librerías de DNA generadas han permitido identificar y caracterizar:

- Dos secuencias repetidas en tándem de la región pericentromérica del cromosoma X.
- Un retrotransposón pericentromérico.
- Un satélite centromérico presente en todos los cromosomas del complemento regular.

2. Se han analizado los patrones de fosforilación de la histona H3 en los residuos Serina 10, Serina 28, Treonina 4 y Treonina 11 durante la meiosis masculina en *S. ocellaris* y *S. coprophila*.

- Durante la meiosis I, la condensación cromosómica y la fosforilación de H3 difiere entre cromosomas de distinto origen parental.
- Durante la meiosis II, y en contraste con el resto de cromosomas, no se detecta H3 fosforilada en la región centromérica del cromosoma X materno.
- Se propone que la falta de histona H3 fosforilada en la región centromérica del cromosoma X materno inactiva la función centromérica de dicho cromosoma en meiosis II.

3. Se ha microdiseccionado y microclonado la región telomérica 1A del cromosoma IV de *S. coprophila*. La librería de DNA generada ha permitido identificar y caracterizar una secuencia telomérica específica de este cromosoma.