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FISH analysis of *Drosophila melanogaster* heterochromatin using BACs and *P* elements

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Abstract The heterochromatin of chromosomes 2 and 3 of *Drosophila melanogaster* contains about 30 essential genes defined by genetic analysis. In the last decade only a few of these genes have been molecularly characterized and found to correspond to protein-coding genes involved in important cellular functions. Moreover, several predicted genes have been identified by annotation of genomic sequence that are associated with polytene chromosome divisions 40, 41 and 80 but their locations on the cytogenetic map of the heterochromatin are still uncertain. To expand our current knowledge of the genetic functions located in heterochromatin, we have performed fluorescence in situ hybridization (FISH) mapping to mitotic chromosomes of nine bacterial artificial chromosomes (BACs) carrying several predicted genes and of 13 *P* element insertions assigned to the proximal regions of 2R and 3L. We found that 22 predicted genes map to the h46 region of 2R and eight map to the h47 regions of 3L. This amounts to at least 30 predicted genes located in these heterochromatic regions, whereas previous studies detected only seven vital genes. Finally, another 58 genes localize either in the euchromatin-heterochromatin transition regions or in the proximal euchromatin of 2R and 3L.

gene density; (v) low genetic recombination; (vi) enrichment in repetitive sequences.

Heterochromatin has been traditionally regarded as a genomic “wasteland”, but several studies indicate that in *Drosophila melanogaster* this peculiar genomic component performs various important biological functions such as gene regulation, centromere and telomere function and meiotic chromosome transmission (reviewed by Gatti and Pimpinelli 1992; Wheeler and Wakimoto 1995; Zuckerkandl and Hennig 1995; Dimitri 1997; McKee 1998; Eissenberg and Hilliker 2000; Sullivan et al. 2001). In addition, detailed genetic analyses have defined about 30 essential genes required for viability that map to the heterochromatin of chromosomes 2 and 3 (Hilliker 1976; Marchant and Holm 1988; Dimitri 1991; Koryakov et al. 2002; Tulin et al. 2002). They are not uniformly distributed throughout the heterochromatin of mitotic cells (Dimitri 1991; Koryakov et al. 2002; see map in Fig. 1), most of them being located in 4',6-diamino-2-phenylindole (DAPI)-dull fluorescent regions. For example, on chromosome 2, *l(2)41Aa* and *rolled* map to region h41; *l(2)41Ad* is located in h43–h44; and eight loci on the left arm [i.e., *l(2)40Fa*, *l(2)40Fc*, *concertina*, *light*, *l(2)40Fd*, *l(2)40Fe*, *l(2)40Ff* and *l(2)40Fg*] and four on the right arm [i.e., *l(2)41Ae*, *l(2)41Af*, *l(2)41Ah* and *Nipped-B*] are grouped within h35 and h46, respectively. The h35 and h46 regions are the most distal portions of mitotic heterochromatin of chromosome 2, encompassing roughly 2 Mb and 1.5 Mb of DNA, respectively. In addition to genes, these regions harbor various clusters of transposable elements (TEs) and are devoid of satellite DNAs (Carmena and Gonzales 1995; Pimpinelli et al. 1995; Dimitri 1997). The early prediction that essential genes located in heterochromatin correspond to unique sequences (Hilliker 1976) has been confirmed by molecular analyses. At least *light*, *concertina*, *rolled* and *Nipped-B*, in chromosome 2, and *l(3)80Fh*, *l(3)80Fi*, *l(3)80Fj* and *Parp*, in chromosome 3, are single-copy genes (Devlin et al. 1990; Parks and Weischaus 1991; Biggs et al. 1994; Rollins et al. 1999; Schulze et al. 2001;

Introduction

Constitutive heterochromatin is a ubiquitous component of eukaryotic genomes and exhibits similar unusual properties in animals and plants (John 1988). These properties include: (i) compact state throughout most of the cell cycle; (ii) late replication during S-phase; (iii) pericentromeric and/or peritelomeric location; (iv) low

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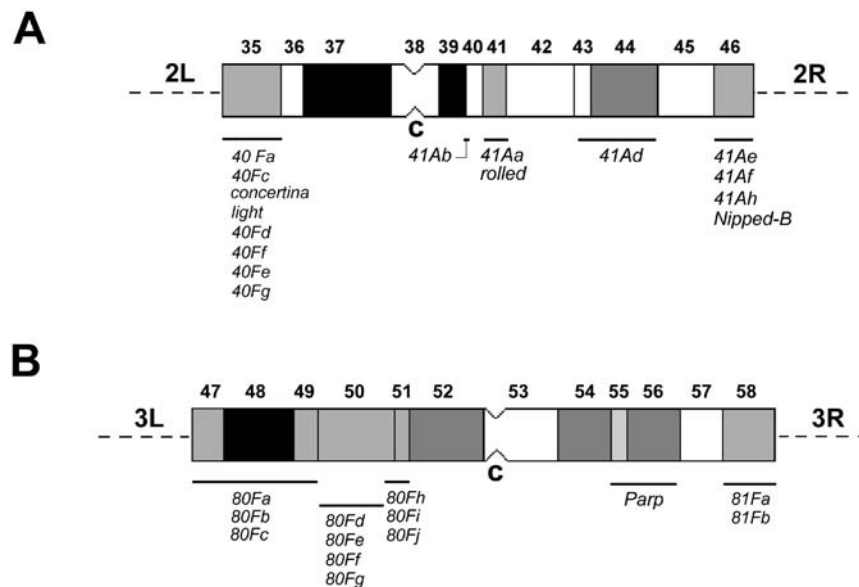


Fig. 1 Diagrams showing the location of vital genes within the heterochromatin of chromosomes 2 (A) and 3 (B) of *Drosophila melanogaster*. Staining with 4',6-diamino-2-phenylindole (DAPI) is a general indicator of AT-richness along the chromosomes (Sumner 1990) and differentiates mitotic heterochromatin into several regions with different degrees of fluorescence (Gatti et al. 1994).

Black and hues of gray correspond to the intensity of DAPI staining, the black regions being the brightest. Numbers mark the heterochromatic regions of the mitotic map of chromosomes 2 and 3 (Dimitri 1991; Koryakov et al. 2002). (2L left arm of chromosome 2; 2R right arm of chromosome 2. 3L left arm of chromosome 3; 3R right arm of chromosome 3. C centromeric region)

Table 1 The known vital genes located in the heterochromatin of chromosome 2. All the genes were identified by conventional genetic analysis before the release of the *Drosophila melanogaster* genome sequence. Molecular identification is only available for *light*, *concertina*, *rolled* and *Nipped-B*

Gene	Mitotic map	Size	Function	Overlaps
<i>l(2)40Fa</i>	h35		Unknown	–
<i>l(2)40Fc</i>	h35		Unknown	–
<i>light</i>	h35	16 kb	Cellular-protein trafficking	AE002734
<i>concertina</i>	h35	10.5 kb	Gastrulation	AE002743
<i>l(2)40Fd</i>	h35		Unknown	–
<i>l(2)40Fe</i>	h35		Unknown	–
<i>l(2)40Ff</i>	h35		Unknown	–
<i>l(2)40Fg</i>	h35		Unknown	–
<i>l(2)41Ab</i>	h39–40		Unknown	–
<i>l(2)41Aa</i>	h41		Chromosome condensation	–
<i>rolled</i>	h41	60 kb	Signal transduction pathway	AABU01001947; AE003090 and AE002642
<i>l(2)41Ad</i>	h43–h44		Legs and wing morphogenesis	–
<i>l(2)41Ae</i>	h46		Unknown	–
<i>l(2)41Af</i>	h46		Unknown	–
<i>l(2)41Ah</i>	h46		Unknown	–
<i>Nipped-B</i>	h46	39 kb	Chromosomal adherin	2R. wgs3 centr. extens.; AE003040

Tulin et al. 2002). A peculiar feature of these genes is that they exhibit a high density of TE-related sequences within their intronic and flanking regions (Devlin et al. 1990; Tulin et al. 2002; Dimitri et al. 2003).

The heterochromatic genes of chromosomes 2 and 3 are involved in important cellular processes (Table 1). For example, on chromosome 2, *light* controls vacuole organization and biogenesis (Warner et al. 1998), *concertina* encodes a maternal α -like subunit of a G protein essential for gastrulation (Parks and Weischaus 1991), the *rolled* product is a mitogen-activated protein kinase required in the *sevenless* signal transduction pathway (Biggs et al. 1994), which may have a role in mediating

the spindle integrity checkpoint (Inoue and Glover 1998), while the *Nipped-B*-encoded protein is homologous to a family of adherins that may play a role in chromosome structure (Rollins et al. 1999). Among the proximal heterochromatic genes of chromosome 2 that have not been cloned yet, *l(2)41Aa* has been suggested to be required for proper chromosome condensation (Cenci et al. 2003) and *l(2)41Ad* may be involved in leg and wing morphogenesis (Dimitri et al. 2003).

The annotation of the *D. melanogaster* genome sequence has predicted several protein-coding genes associated with the proximal division 20, 40, 41 and 80 of polytene chromosomes (Adams et al. 2000; Hoskins et

al. 2002; Misra et al. 2002; GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>). Thus far, these gene annotations have been incorporated into FlyBase with inferred locations on the cytogenetic map of the salivary gland polytene chromosome (<http://www.fruitfly.org/sequence/faq.html#seq-14>) and their mapping in the mitotic heterochromatin remains uncertain. These observations raise some important questions: how many of the predicted genes are actually located in the constitutive heterochromatin of chromosomes 2 and 3? Do some of these correspond to the essential genes identified by genetic analysis? To address these questions nine bacterial artificial chromosomes (BACs) from the proximal regions of 2R and 3L, which contain predicted genes, have been mapped by fluorescence in situ hybridization (FISH) to mitotic chromosomes. This analysis has been integrated by mapping 13 single *P* element insertions. The results show that at least 30 predicted genes are embedded in the most distal regions of constitutive heterochromatin; of these, 22 map to the h46 region of 2R and eight map to the h47 region of 3L.

Materials and methods

Drosophila strains

Genetic markers, mutations and balancer chromosomes are described in Lindsley and Zimm (1992) and FlyBase (<http://fly.ebi.ac.uk:7081/>). Cultures were maintained at 25°C on standard cornmeal-sucrose-yeast-agar medium.

Predicted genes associated with proximal regions of the arms

Predicted genes of the chromosome 2R and of 3L proximal regions were considered in the present study (Tables 2, 3). On 2R, the scaffolds AE002751.2 and AE002760.2 and AE003040, which contained at least 15 genes, have recently been incorporated into a larger scaffold (Release 3) designated 2R_wgs3 centromere extension (Table 2; Hoskins et al. 2002; see also http://www.fruitfly.org/cgi-bin/annot/gbrowse?name=2R_wgs3_centromere_extension); AE003788.4 carries one gene, AE003787.4 contains 13 genes, AE003786.4 carries eight genes and, finally, 21 genes are present in AE003785.4 (Table 2; see also <http://www.fruitfly.org/cgi-bin/annot/basic.pl?qp=queryresults&qtype=basic&datatype=annotation&subset=all;&sessionid=07775>). More than half of these genes are distributed in various sequenced BACs assigned to polytene regions 41–42A (see Table 2; FlyBase <http://flybase.bio.indiana.edu/> and GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>). On 3L, scaffolds AE003599.2, AE002786.2, AE002656.3 and AE002665.4 contain 23, three, three and one genes, respectively (Table 3; see also <http://www.fruitfly.org/cgi-bin/annot/basic.pl?qp=queryresults&qtype=basic&datatype=annotation&subset=all;&sessionid=07892>). These genes are distributed in three sequenced BACs assigned to polytene divisions 79E–80B (Table 3).

P element insertions

19.74.3 and 47.122.1 are *white*⁺ elements (Hagstrom et al. 1996). *P*{wAR}41A, *P*{lacW}l(2)k08138, *P*{lacW}l(2)k07312, *P*{lacW}l(2)k03002, *P*{lacW}k07812, *P*{lacW}k02601, and *P*{lacW}vlck01109 are *lacZ*⁺ and *white*⁺ elements (Bier et al. 1989). *P*{PZ}l(2)02047, *P*{PZ}CH2-6 and *P*{PZ}CH2-1 are marked with *lacZ*⁺ and *rosy*⁺ (Karpen and Spradling 1992). *KG01086* is a

yellow⁺ and *white*⁺ *SUPor-P* element insertion (Roseman et al. 1995).

Cytology

Mitotic chromosome preparations and FISH procedures were described in Gatti et al. (1994). The BAC DNA probes were labeled using a rhodamine nick-translation kit (Boehringer, Mannheim) as described in Dimitri (2003). The Car 20 and pW8 DNA clones, used as probes for *PZ* elements and *Pw*⁺, respectively, were labeled with a biotin nick-translation kit (Boehringer, Mannheim).

Preparation of BAC DNA

Extraction of BAC DNA was as follows: a bacterial culture was seeded in 10 ml of LB medium with antibiotic (20 µg/ml chloramphenicol). Two milliliters of the overnight culture were transferred to a 2 ml microcentrifuge tube and centrifuged for 50 s at 13,000 rpm. The supernatant was discarded and the bacterial pellet resuspended in 100 µl of 50 mM TRIS-HCl, pH 8.5, 10 mM EDTA buffer. Then 200 µl fresh 0.2 M NaOH, 1% SDS solution was added. The tubes were inverted to mix and immediately 150 µl of 7.5 M ammonium acetate solution was added. The tubes were again inverted to mix. After centrifugation at 13,000 rpm for 15 min in a microcentrifuge, the supernatant was transferred to a 1.5 ml microcentrifuge tube with 300 µl of isopropanol. After mixing by inversion, the preparation was centrifuged at 13,000 rpm for 10 min in a microcentrifuge. The supernatant was removed. The pellet and the walls of the tube were washed with 300 µl of 70% ethanol; the preparation was then centrifuged at 13,000 rpm for 5 min, the supernatant removed and the pellet air-dried. The pellet was resuspended in 50 µl of 10 mM TRIS-HCl, pH 8, 1 mM EDTA buffer containing 50 µg/ml RNase A. This was followed by incubation at 65°C for 10 min. The DNA was stored at 4°C for short-term use.

Microphotography

Chromosome preparations were analyzed using a computer-controlled Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics). Fluorescence was visualized using the Pinkel No1 filter set combination (Chroma Technology). The fluorescent signals were recorded by IP Spectrum Lab Software and edited with Adobe PhotoShop 5.

Results

Mapping of BACs to mitotic chromosomes by FISH

We undertook FISH to mitotic chromosomes of *y; cn bw sp* and/or Oregon R strains of *D. melanogaster* to map nine BACs that originated from polytene regions 41–42A of 2R and 79E–80B of 3L. It is important to point out that most of these BACs also contain variable amounts of sequences related to TEs. A priori, the intensity and number of hybridization signals may be expected to reflect several factors including the copy number and arrangement of TE sequences within the genome, as well as their relative concentration within a given BAC. Experimentally, however, we found that each tested BAC produced a prominent fluorescent signal on 2R or 3L, the mapping of which was consistent with the BAC location on polytene chromosomes (see below). Only in a few

Table 2 Predicted genes located in polytene regions 41–42A. The scaffold sequence 2R. wgs3 centromere extension from Release 3 (Hoskins et al. 2002; GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>) contains three smaller Release 2 scaffolds, AE002751, AE002760.2 and AE003040. The polytene locations refer to the in situ hybridization mapping of bacterial artificial chromosomes (BACs) performed by BDGP (R. Hoskins, personal communication; GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>) with the exception of BACR23M07, shown in parenthesis, which we have mapped to 42A. (CC, chromo-center)

Gene	Overlaps	Polytene map
<i>CG40285</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>CG17528</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>CG12547</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>CG17082</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>CG40287</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>CG40282</i>	2R. wgs3 centr. extens. BACR01012	CC
<i>Nipped-B</i> ^{a, b}	2R. wgs3 centr. extens. BACR01012	CC
<i>CG17883</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG17478</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG17486</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>p120ctn</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG40290</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG40279</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG40293</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>CG40278</i>	2R. wgs3 centr. extens. BACR15A11	CC
<i>TpnC41C</i>	AE003788 BACR11B14	41D1–E2
<i>CG3107</i>	AE003787 BACR11B14	41D1–E2
<i>CG30442</i>	AE003787 BACR11B14	41D1–E2
<i>CG2944</i>	AE003787 BACR11B14;BACR06P07	41D1–E2;
<i>CG3136</i>	AE003787 BACR11B14;BACR06P07	41D1–E2; 41E1–3
<i>CG14768</i>	AE003787 BACR11B14;BACR06P07	41D1–E2; 41E1–3
<i>CG2905</i>	AE003787 BACR11B14;BACR06P07	41D1–E2; 41E1–3
<i>d4</i>	AE003787 BACR11B14;BACR06P07	41D1–E2; 41E1–3
<i>Ogt</i>	AE003787 BACR32F22	41F1–2
<i>CG10465</i>	AE003787 BACR32F22	41F1–2
<i>CG10395</i>	AE003787 BACR32F22	41F1–2
<i>CG30441</i>	AE003787 BACR32F22	41F1–2
<i>CG10396</i>	AE003787 BACR32F22	41F1–2
<i>CG10417</i>	AE003787 BACR32F22	41F1–2
<i>CG30437</i>	AE003786 BACR32F22	41F1–2
<i>CG32838</i>	AE003786 BACR32F22	41F1–2
<i>CG30440</i>	AE003786 BACR32F22	41F1–2
<i>CG30438</i>	AE003786 BACR04B08	41F1–3
<i>CG12408</i>	AE003786 BACR04B08	41F1–9
<i>CG17510</i>	AE003786 BACR27C13	41F1–9
<i>CG17508</i>	AE003786 BACR27C13	41F1–9
<i>CG11665</i>	AE003786 BACR27C13	41F1–9
<i>CG1344</i>	AE003785 BACR27C13	41F1–9
<i>CG8426</i>	AE003785 BACR27C13	41F1–9
<i>CG1294</i>	AE003785 BACR27C13	41F1–9
<i>CG8245</i>	AE003785 BACR27C13	41F1–9
<i>CG1298</i>	AE003785 BACR27C13	41F1–9
<i>CG11066</i>	AE003785 BACR27C13	41F1–9
<i>CG17337</i>	AE003785 BACR27C13	41F1–9
<i>vulcan</i> ^{a, b}	AE003785 BACR27C13	41F1–9
<i>apterous</i> ^{a, b}	AE003785 BACR27C13	41F1–9
<i>CG12792</i>	AE003785 BACR45O18	41F6–42A1
<i>CG7791</i>	AE003785 BACR45O18	41F6–42A1
<i>gp210</i>	AE003785 BACR45O18	41F6–42A1
<i>CG11163</i>	AE003785 BACR45O18	41F6–42A1
<i>Or42a</i>	AE003785 BACR45O18	41F6–42A1
<i>Tsp42A</i>	AE003785 BACR45O18	41F6–42A1
<i>Or42b</i>	AE003785 BACR45O18	41F6–42A1
<i>CG7796</i>	AE003785 BACR35F09;BACR23M07	42A3–16;42A
<i>CG14469</i>	AE003785 BACR35F09;BACR23M07	42A3–16;42A
<i>CG30436</i>	AE003785 BACR35F09;BACR23M07	42A3–16;42A
<i>CG12551</i>	AE003785 BACR35F09;BACR23M07	42A3–16;42A
<i>CG14470</i>	AE003785 BACR35F09;BACR23M07	42A3–16;42A

^a Genes with known mutant alleles

^b Previously identified by conventional genetic analysis

cases did we observe additional minor hybridization sites. It is also important to emphasize that the BACs tested gave similar mapping results in both the *y*; *cn bw sp* and the Oregon R strain.

Bacterial artificial chromosomes from polytene divisions 41–42A

The results of this analysis are shown in Figs. 2, 3. We initially tested BACR11B14, BACR06P07 and BACR32F22 (Table 2). At the molecular level,

Table 3 Predicted genes in chromosomes 3 located at 79E–80B. A number of predicted genes previously annotated in Release 2 have been eliminated in the recent Release 3 and therefore are not listed in the table. The polytene locations refer to the in situ hybridization mapping of BACs performed by BDGP (R. Hoskins, personal communication; GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>)

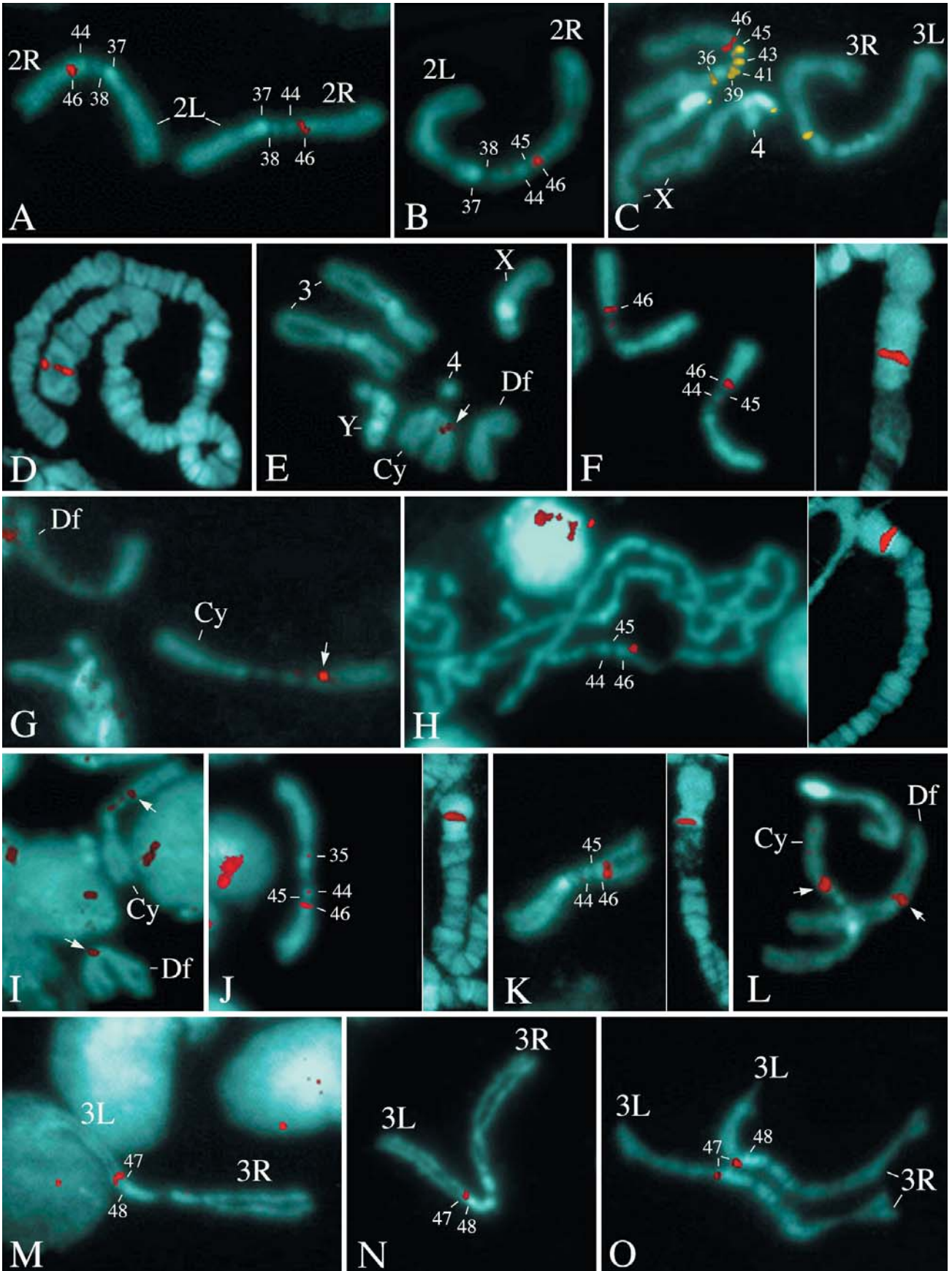
Gene	Overlaps	Polytene map
<i>jim</i>	AE003599 BACR22021	79E3–F2
<i>CG11226</i>	AE003599 BACR48E05	79E6–80A2
<i>CR32456</i> (tRNA)	AE003599 BACR48E05	79E6–80A2
<i>CR32460</i> (tRNA)	AE003599 BACR48E05	79E6–80A2
<i>CG32462</i>	AE003599 BACR48E05	79E6–80A2
<i>CG32459</i>	AE003599 BACR48E05	79E6–80A2
<i>CG13239</i>	AE003599 BACR48E05	79E6–80A2
<i>CG33169</i>	AE003599 BACR48E05	79E6–80A2
<i>CG33170</i>	AE003599 BACR48E05	79E6–80A2
<i>CG11137</i>	AE003599 BACR48E05	79E6–80A2
<i>Arf79F</i>	AE003599 BACR48E05	79E6–80A2
<i>snoRNA:21</i>	AE003599 BACR48E05	79E6–80A2
<i>CG11109</i>	AE003599 BACR48E05	79E6–80A2
<i>CG10712</i> ^a	AE003599 BACR48E05	79E6–80A2
<i>Ssl1</i>	AE003599 BACR48E05	79E6–80A2
<i>CG11128</i> ^a	AE003599 BACR48E05	79E6–80A2
<i>CG11131</i>	AE003599 BACR48E05	79E6–80A2
<i>CG11133</i>	AE003599 BACR48E05	79E6–80A2
<i>CG12768</i>	AE003599 BACR48E05	79E6–80A2
<i>CG11100</i> ^a	AE003599 BACR48E05	79E6–80A2
<i>CG32461</i>	AE003599 BACR48E05	79E6–80A2
<i>CG32457</i>	AE003599 BACR48E05;BACR17M18	79E6–80A2;80A1–B1
<i>nrm</i> ^b	AE003599 BACR48E05;BACR17M18	79E6–80A2;80A1–B1
<i>CG32458</i>	AE003599 BACR17M18	80A1–B1
<i>Qm</i>	AE002786 BACR17M18	80A1–B1
<i>CkIIalpha</i>	AE002786 BACR17M18	80A1–B1
<i>CG32350</i>	AE002786 BACR17M18	80A1–B1
<i>CG32230</i>	AE002665 BACR22B15	80B1–2
<i>alpha-Cat</i>	AE002665 BACR22B15	80B1–2
<i>CG32231</i>	AE002665 BACR22B15	80B1–2
<i>nAcRalpha-80B</i>	AE014189 BACR22B15	80B1–2

^a Genes with known alleles generated by gene disruption projects

^b Previously identified by conventional genetic analysis

BACR06P07 overlaps proximally with BACR11B14 and its distal border is very close to the proximal end of BACR32F22; together, these BACs identify 17 predicted genes and cover a DNA region of about 500 kb that extends through the scaffolds AE003788.3, AE003787.3 and AE003786.3 (Gadfly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>). As shown in Fig. 2A, B, a strong signal given by BACR11B14 clearly maps to h46. This mapping was further investigated by FISH experiments in which both BACR11B14 and AAGAG satellite DNAs were simultaneously hybridized to mitotic chromosomes. The tandemly repeated AAGAG sequences form large DNA blocks, corresponding to h36, h38, h40, h43 and h45 n-banded regions of chromosome 2 (Dimitri 1991; Lohe et al. 1993). The fluorescent intensity of the AAGAG signal is usually quite strong in h45 and may slightly spread over the flanking regions. Thus, a putative location of BACR11B14 in the proximal portion of h46 is expected to produce a hybridization signal very close to or even partly overlapping that of AAGAG repeats in h45. On the other hand, if BACR11B14 was located in the middle-distal part of h46, a region of separation between AAGAG and BACR11B14 signals would be seen. As shown in Fig. 2C, the AAGAG hybridization signal in h45 is clearly separated from that of BACR11B14 in h46. This observation suggests that this BAC maps to the middle-distal part of h46. The location of BACR06P07 is

Fig. 2A–O Fluorescence in situ hybridization (FISH) mapping of bacterial artificial chromosome (BAC) sequences to the heterochromatin of chromosomes 2 and 3. Chromosomes are stained with DAPI and pseudocolored in *blue*; BAC fluorescent hybridization signals are pseudocolored in *red*. A prominent BACR11B14 signal maps to h46 of the Oregon R (**A**) and *cn bw sp* (**B**) strains; simultaneous FISH with BACR11B14 (*red*) and AAGAG DNA probes pseudocolored in *yellow* (**C**); all the expected AAGAG sites are distinguishable on the heterochromatin of chromosome 2 (h36, h38, h40; h43 and h45); the signal in h45 is separated from that of BACR11B14 in h46. On polytene chromosomes BACR11B14 maps to 41D (**D**). The BACR11B14 hybridization signal is present in h46 (*arrow*) of the *Cy* balancer and is absent in *Df(2R)MS41A10* (*Df*) (**E**). The BACR06P07 signal maps to h46 on mitotic chromosomes (*left panel*) and to 41D on polytene chromosomes (*right panel*) (**F**). The BACR06P07 signal of h46 (*arrow*) is present in the *Cy* balancer and is absent in *Df(2R)MS41A10* (*Df*) (**G**). The BACR32F22 signal maps at the base of h46 (*left panel*) and to region 41E (*right panel*) of polytene chromosomes (**H**). *Df(2R)MS41A10* (*Df*) and the *Cy* balancer exhibit the prominent BACR32F22 signal (*arrow*) at the base of h46 (**I**). The BACR01012 signal maps to the proximal part of region h46 (*left panel*) and is located in 41C–D of polytene chromosomes (*right panel*) (**J**). BACR23M07 is not included in region h46 (*left panel*); on polytene chromosomes a prominent signal of this BAC is located in region 42A (*right panel*) (**K**). *Df(2R)MS41A10* (*Df*) and *Cy* chromosomes exhibit a BACR23M07 signal (*arrow*) (**L**). The BACR48E05 signal falls outside region h47 (**M**). BACR17M18 (**N**) and BACR22B15 map to h47 (**O**)



cytologically very similar to that of BACR11B14 (Fig. 2F), although at the molecular level the former BAC extends distally about 100 Kb more than the latter (GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>). In contrast, BACR32F22 exhibits a more distal location in that it lies at the base of region h46. On polytene chromosomes, we mapped BACR11B14 (Fig. 2D) and BACR06P07 (Fig. 2F) to region 41D, while BACR32F22 was located in 41E (Fig. 2H). The polytene location of these BACs differs slightly from that determined by the BDGP (Hoskins, personal communication; see Table 2). We also mapped BACR01012, which does not overlap with the other tested BACs of 2R and contains the genomic regions of *Nipped-B* and other predicted genes from Release 3 (scaffold 2R. wgs3 centromere extension; see Table 2). As shown in Fig. 2J, the BACR01012 probe gives three distinct hybridizations signals on 2Rh: a prominent site maps to the very proximal portion of h46, whereas two minor ones are located in h44 and h35. The relatively higher intensity of the signal located in h46 is consistent with our previous mapping of *Nipped-B* in this region (Dimitri et al. 2003). As anticipated above, the weaker signals in h44 and h35 are likely to reflect the hybridization of repetitive sequences that are known to be present in such regions (Pimpinelli et al. 1995). On polytene chromosomes we have mapped a single sharp signal of BACR01012 to division 41C–D (Fig. 2J). Fluorescence in situ hybridization on mitotic chromosomes with BACR35F09 and BACR23M07 clearly showed that these BACs are excluded from h46 and thus are likely to be located in euchromatin (Fig. 2K). This is in agreement with our polytene chromosome mapping showing that BACR35F09 and BACR23M07 lie in the euchromatic region 42A (Fig. 2K).

Further evidence supporting these results was obtained by FISH mapping of the same BACs in strains carrying two overlapping deficiencies, i.e., *Df(2R)M41A10* and *Df(2R)M41A4*. *Df(2R)M41A10* removes approximately the entire mitotic heterochromatin of 2R including h46, while *Df(2R)M41A4* only lacks h46 (Dimitri 1991). On polytene chromosomes, *Df(2R)M41A10* lacks regions 41A–41D of 2R, while *Df(2R)M41A4* is deleted for regions 41D–41F (Koryakov et al. 1996). Genetically, both chromosomes fail to complement mutations in genes located in h46 (Fig. 1). The results show that *Df(2R)M41A10* lacks the BACR11B14 (Fig. 2E), BACR06P07 (Fig. 2G) and BACR01012 (data not shown) prominent hybridization signal located in h46, while exhibiting that given by BACR32F22 (Fig. 2I), BACR23M07 (Fig. 2L) and BACR35F09 (data not shown). *Df(2R)M41A4* lacks the h46 signal of BACR11B14, BACR06P07, BACR01012 and BACR32F22 while retaining those of BACR35F09 and BACR23M07 (data not shown). Together, these observations confirm that BACR11B14, BACR06P07 and BACR01012 originate from region h46, whereas BACR35F09 and BACR23M07 are euchromatic. Furthermore, the inclusion of BACR32F22 in *Df(2R)M41A4*,

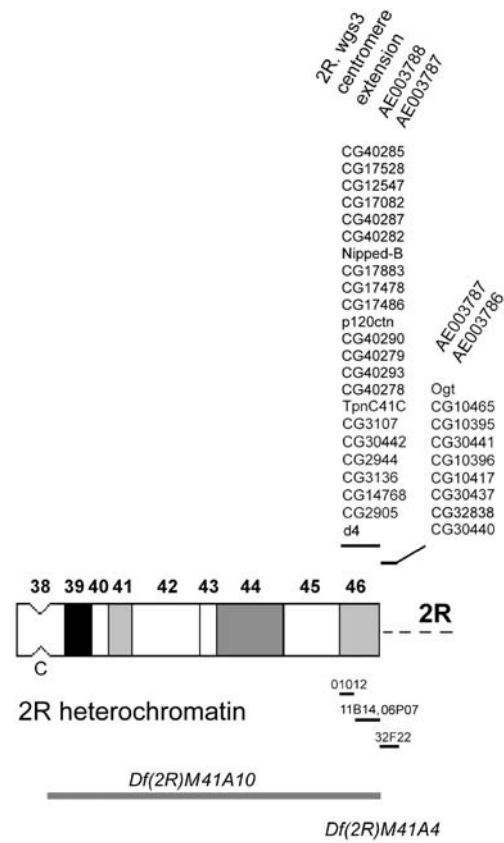


Fig. 3 Mapping of BACs and predicted genes to the heterochromatin of chromosome 2. Diagram showing the mapping of predicted genes to the heterochromatin of the right arm of chromosome 2 (above). In addition to vital gene *Nipped-B*, at least 22 predicted genes are likely to be located at the euchromatin-heterochromatin junction of 2R. The gray lines represent the extent of *Df(2R)M41A10* and *Df(2R)M41A4* (below)

but not in *Df(2R)M41A10*, suggests that this BAC is not located in h46.

Bacterial artificial chromosomes from polytene divisions 79E–80B

We next analyzed three gene-carrying BACs, i.e., BACR48E05, BACR17M18 and BACR22B15, which have been mapped to divisions 79E6–80B1 of 3L by BDGP (Table 3). BACR48E05 is within scaffold AE003599, while BACR17M18 is contained in AE003599 and AE002786. These two BACs partly overlap and extend along polytene regions 79E6–80A2 and 80A1–B1, respectively. BACR48E05 carries 22 genes (from *CG11226* to *nrm*), while BACR17M18 contains six genes, two of which (*CG32457* and *nmr*) are also present in BACR48E05. BACR22B15, which maps to divisions 80B1–2, derives from scaffolds AE002665 and AE014189 and contains four genes. The results of FISH mapping experiments are shown in Figs. 2,

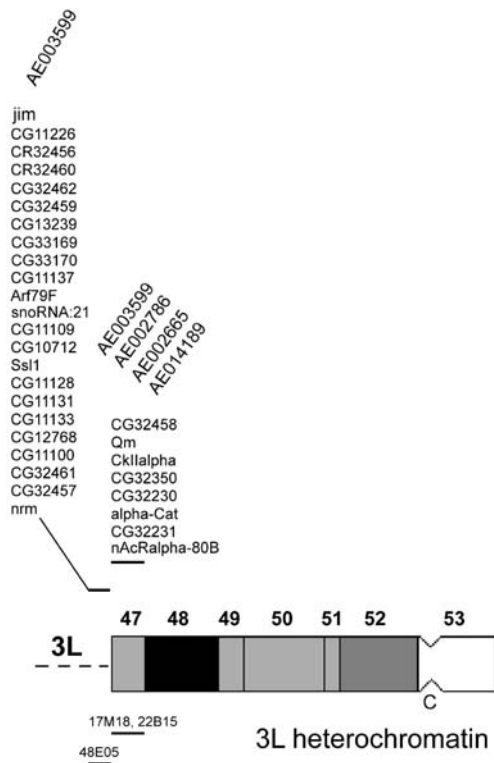


Fig. 4 Mapping of BACs and predicted genes to the heterochromatin of chromosome 3. Diagram showing the mapping of predicted genes to the heterochromatin of the left arm of chromosome 3 (above). At least eight predicted genes are located in region h47. A large group of 23 predicted genes are distributed throughout the proximal euchromatin and the euchromatin-heterochromatin junction of 3L. The *jim* gene has been included in this group because, although it is located in the neighboring BACR22021, molecularly it is very close to *CG11226* (about 30 kb) and is present in the same scaffold

4. A prominent hybridization signal of BACR48E05 maps distally to h47, a DAPI-dull fluorescence region of 3L h. This suggests that this BAC spans a region corresponding to the euchromatin-heterochromatin junction and/or to proximal euchromatin (Fig. 2M). In contrast, the signals of BACR17M18 and BACR22B15 clearly map to h47, the most distal region of the mitotic heterochromatin of 3L (Fig. 2N, O).

Characterization of single *P* element insertions

Our analyses have been integrated by mapping 13 *P* element insertions assigned to polytene region 41 of chromosome 2 (Table 4; see also Materials and methods). Most insertions were previously mapped on polytene chromosomes only (see FlyBase 2003 <http://flybase.bio.indiana.edu/>), except *CH2-6* and *CH2-1*, which were also localized on mitotic chromosomes (Zhang and Spradling 1994).

Table 4 Single *P* element insertions mapped to the heterochromatin of chromosome 2. *19.74.3* and *47.122.1* were mapped to polytene chromosome by M. Martin (unpublished); the polytene mapping data of the other inserts are taken from FlyBase (2003 <http://flybase.bio.indiana.edu/>). (CC, chromocenter; n.d., not detected)

Insert	Polytene map	Mitotic map
19.74.3	CC	h40-h41
47.122.1	CC	h41
<i>PwAR41A</i>	41A	h44
<i>P{PZ} CH2-6</i>	n.d.	h44
<i>P{lacW}l(2)k07312b</i>	41C-41C	h45-h46
<i>P{PZ} CH2-1</i>	CC	h46
<i>P{lacW}l(2)k03002</i>	41C-41C	h45-h46
<i>KG01086</i>	n.d.	h46
<i>P{lacW}k07812a</i>	41C-41C	h46
<i>P{PZ} l(2)02047 (Nipped-B)</i>	41C1-41C6	h46
<i>P{lacW}k02601 (Nipped-B)</i>	41D-41D	h46
<i>P{lacW}l(2)k01109 (vulcan)</i>	41F8-41F9	Base of h46
<i>P{lacW}l(2)k08138</i>	41F8-41F9	Base of h46

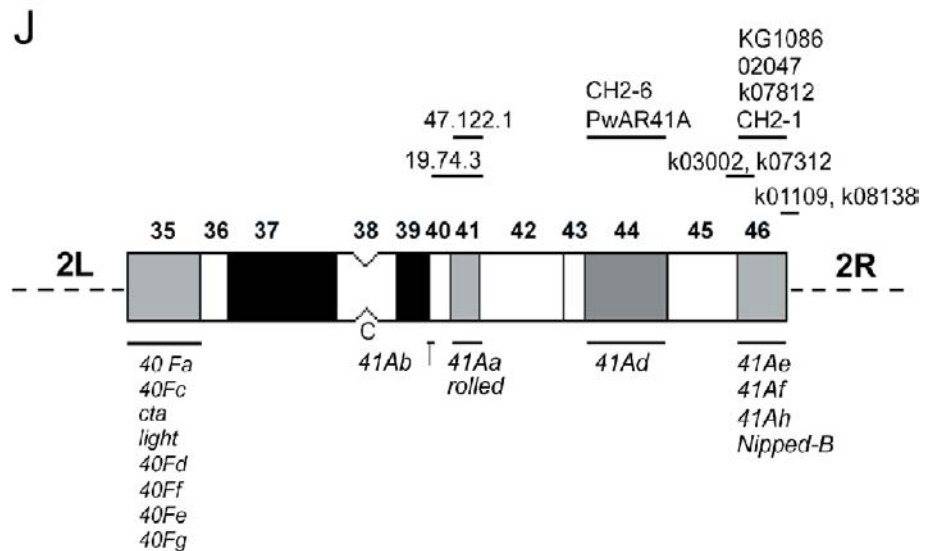
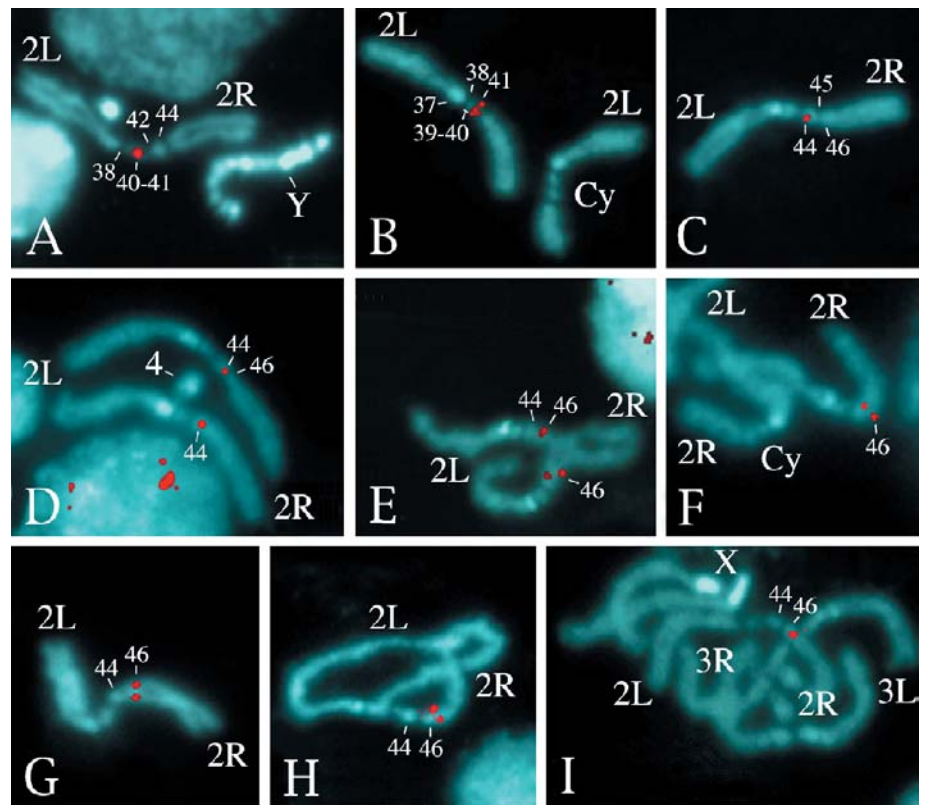
Mapping by FISH

The results of this analysis are shown in Fig. 5A-J and Table 4. It appears that four inserts (*19.74.3*, *47.122.1*, *P{wAR}41A* and *CH2-6*) map to the proximal heterochromatic regions of 2R, while the remaining inserts are located in distal portions of 2R heterochromatin. *19.74.3* is the most proximal insert within 2R and lies in h40-h41 (Fig. 5A); *47.122.1* maps to h41 (Fig. 5B), consistent with our previous deficiency mapping and FISH localization of *rolled* (Dimitri 1991; Berghella and Dimitri 1996). Both *CH2-6* and *P{wAR}41A* map to h44 (Fig. 5C,D). *CH2-6* was previously mapped to h42-h44 (Zhang and Spradling 1994) and here we provide a more detailed localization. Among the distally located insertions, *P{lacW}l(2)k03002* and *P{lacW}l(2)k07312* map to h45-h46, while *P{lacW}l(2)k07812*, *CH2-1*, *P{PZ}l(2)02047*, *P{lacW}l(2)k02601* and *KG01086* are located in h46 (Fig. 5E-G, Table 4). Finally, *P{lacW}l(2)k01109* and *P{lacW}l(2)k08138* map at the base of h46 (Fig. 5H, I).

Genetic tests

Four of the lines examined are homozygous viable (*19.74.3*, *P{wAR}41A*, *KG01086* and *CH2-1*), whereas the remainder are recessive lethals. In particular, *l(2)02047* and *l(2)02601* are lethal alleles of *Nipped-B* (Rollins et al. 1999); *l(2)k01109* is a lethal allele of *vulcan*, a gene located at 41F, and *l(2)k08138* is associated with the predicted gene *CG12792* (<http://fly.ebi.ac.uk:7081/bin/fbidq.html?FBgn0022288>), which also lies at 41F distally to *vulcan* (Table 2). In order to establish whether the lethality associated with the inserts was caused by the disruption of genes located in the constitutive heterochromatin of 2R, lines *47.122.1*, *CH2-6*, *l(2)k07312*, *l(2)k03002*, *l(2)k07812*, *l(2)02047*, *l(2)02601*, *l(2)k01109* and *l(2)k08138*, were tested against *Df(2R)M41A10*. The results showed that *47.122.1* and,

Fig. 5A–J Examples of FISH mapping of *P* element insertions in the heterochromatin of chromosome 2. Chromosomes are stained with DAPI and pseudocolored in blue; fluorescent hybridization signals are pseudocolored in red. The 19.74.3 insert maps at h40-h41 (A); the 47.122.1 signal is located in h41 (B). For *P{wAR}41A* (C) and *CH2-6* (D) a single signal is located in h44 in both lines. *CH2-1* maps to h45-h46 (E). The *Nipped* allele *l(2)02047* (F) and *KG01086* (G) map to h46. *l(2)k01109* (H) and *l(2)k08138* (I) are located at the base of h46. J Diagram showing the distribution of the single *P* element mapped to the heterochromatin of chromosome 2 relative to the known essential genes



as expected, the two *Nipped-B* alleles, *l(2)02047* and *l(2)02601*, failed to complement this *Df(2R)M41A10*, while full complementation was seen with the other tested lines. Further genetic assays using ethylmethane sulfonate-induced lethal alleles of 2R h genes revealed that 47.122.1 is a lethal allele of *rolled*, in agreement with previous observations (M. Muller, unpublished). The lethality of the *l(2)k01109* chromosome is due to a *P* element inserted within the *vulcan* gene, which lies outside *Df(2R)M41A10*. Finally, the lethality of the chromosomes that complement *Df(2R)M41A10*, but carry

a heterochromatic insert located within the h45–h46 portion may be due to other *P* elements present on chromosome 2. This should be the case of *l(2)k07312*, *l(2)k03002*, and *l(2)k07812* chromosomes, each carrying an additional insertion (data not shown).

Mapping of predicted genes to the heterochromatin of 2R and 3L

The FISH analysis of BACs and *P* elements performed in the present study enabled us to map predicted genes to the mitotic heterochromatin of chromosomes 2 and 3 (Figs. 2, 4, Tables 2, 3).

Genes of 2R h

We found that BACR01012, BACR11B14 and BACR06P07 map to h46, BACR01012 being located at the proximal end of this region (Fig. 2). We also obtained some insight into the location of BACR15A11 by FISH mapping of *KG01086*. This *SUP* or *P* element is inserted 2.5 kb upstream of the 5' end of *CG17486* (<http://flypush.imgen.bcm.tmc.edu/pscreen/>), one of the eight genes carried by BACR15A11 (Table 2). As shown in Fig. 5G and Table 4, *KG01086* maps to region h46. This result indicates that BACR15A11 is also included in h46. It then follows that *Nipped-B* and all 22 predicted genes located in the genomic portions covered by BACR01012, BACR15A11, BACR11B14 and BACR06P07 map to region h46. In particular, our mapping of BACR01012 suggests that *Nipped-B* and the six predicted genes carried by this BAC have a more proximal location within h46 than the other genes of the group.

Our experiments showed that BACR32F22, which carries nine predicted genes, is located at the base of region h46, too close to the heterochromatin-euchromatin junction to allow resolution of whether it lies within or outside h46. However, this BAC can be mapped more precisely by comparison between the FISH experiments on *Df(2R)M41A10* and *Df(2R)M41A4* chromosomes: the former deletion lacks approximately the entire mitotic heterochromatin of 2R including h46, while the latter lacks h46 together with portions of proximal euchromatin (see also Results). We found that the prominent signal of BACR32F22 at the base of h46 is present in *Df(2R)M41A10* and absent in *Df(2R)M41A4*. Together, these observations strongly suggest that BACR32F22 maps outside h46 and thus the nine genes carried by this BAC are likely located in the transition region between euchromatin and heterochromatin or in proximal euchromatin. Our analysis was then extended by the mapping of BACR35F09 and BACR23M07, which carry five genes located in the most distal portion of scaffold AE003785. The results clearly show that both BACs, carrying five predicted genes, map to euchromatin (Fig. 2K, L). Finally, the remaining 21 genes (from *CG30438* to *Or42b*) found between the distal end of BACR32F22 and the proximal border of BACR35F09 are also likely to be scattered throughout the transition region and proximal euchromatin. This conclusion is also supported by the finding that *P* element insertions within, or close to, genes of this group [i.e., *l(2)k01109* and *l(2)k08138*] also map outside h46 (Fig. 5, Table 4).

Genes of 3L h

The mapping of 3L h predicted genes is shown in Fig. 4. FISH experiments with BACR17M18 and BACR22B15 showed that eight predicted genes (from *CG32458* to *nAcRalpha-80B*) are located in h47, the most distal region of 3L h (Table 3). By contrast, the mapping of BACR48E05 indicated that 23 predicted genes of scaffold AE003599 (from *CG11226* to *nrm*) are distributed throughout the proximal euchromatin and euchromatin-heterochromatin junction.

Discussion

The results of our analyses indicate that, on chromosome 2, 22 predicted genes map to the h46 region, while, on chromosome 3, eight genes are located within h47 (Figs. 3, 4). This amounts to, at least, 30 predicted genes located in the most distal heterochromatic regions of 2R and 3L, where previous studies only detected seven vital genes [*l(2)41Ae*, *l(2)41Af*, *l(2)41Ah*, *Nipped-B*, *l(3)80Fa*, *l(3)80Fb* and *l(3)80Fc*; Hilliker 1976; Rollins et al. 1999; Dimitri 1991; Dimitri et al. 2003; Koryakov et al. 2002; see Fig. 1]. How might this difference be explained? It is known that some predictions of genes may actually represent portions of the same gene, or may be spurious. This may lead to an overestimate of the actual number of genes in heterochromatin. It is also possible that classical mutagenesis methods might not allow the detection of all the genetic loci present in a given region. This is certainly true for certain regions of *D. melanogaster* euchromatin: for example, the X chromosome polytene band 10A1–2 and the *Adh* gene region were found to contain more genes than those determined by genetic analysis (Kozlova et al. 1997; Ashburner et al. 1999). A similar situation may also exist within heterochromatin; this has been thoroughly discussed by Hoskins et al. (2002).

Four additional predicted genes (*CG40241*, *CG12552*, *CG40239* and *CG4024*) have been detected in the 258 kb scaffold AABU01001947, which contains the large *rolled* gene region (Hoskins et al. 2002). It is conceivable that these genes are also located in region h41 together with *rolled*.

Our mapping studies may also yield an interesting outcome for further study of the relationships between mitotic and polytene chromosome heterochromatin. In particular, we found that all BACs and *P* element inserts located in polytene divisions 41C–41D map to region h46 on mitotic heterochromatin. It is therefore tempting to speculate that, during polytenization, sequences from h46 contribute to the formation of 41C–41D segments of salivary gland chromosomes.

The known genes located in the heterochromatin of *Drosophila* are on average particularly long due to the occurrence of long introns enriched in TE-related DNA sequences (Devlin et al. 1990; Kurek et al. 2000; Sinclair et al. 2000; Carvalho et al. 2002; Dimitri et al. 2003). Similarly, many of the predicted genes that we mapped to

h46 and h47 also have long introns, for example, *CG2905*, dd4 on 2R and *alpha-catenin* on 3L (Hoskins et al. 2002; GadFly 2003 <http://www.fruitfly.org/cgi-bin/annot/query>), but the average density of TEs does not appear to be as high as that found in introns of known heterochromatic genes (Devlin et al. 1990; Kurek et al. 2000; Dimitri et al. 2003). Both predicted and known heterochromatic genes do not seem to have molecular functions that would distinguish them from genes located in euchromatin (Table 1; Devlin et al. 1990; Parks and Weischaus 1991; Biggs et al. 1994; Rollins et al. 1999; Adams et al. 2000; Kurek et al. 2000; Schulze et al. 2001; Hoskins et al. 2002; Tulin et al. 2002). By contrast, heterochromatic genes might have regulatory requirements and expression patterns that are different from those of euchromatic genes (Wakimoto and Hearn 1990; Eberl et al. 1993). It is tempting to speculate that the occurrence of long introns might have a functional influence on the expression of genes located in heterochromatin as suggested by the finding that, in *Caenorhabditis elegans* and *Homo sapiens*, genes expressed at low levels tend to have substantially longer introns than those that are highly expressed (Castillo-Davis et al. 2002).

P element insertions in the heterochromatin of chromosome 2

Insertional mutagenesis with natural or marked *P* elements has accelerated genetic and molecular studies on heterochromatin (Devlin et al. 1990; Karpen and Spradling 1992; Zhang and Spradling 1994; Roseman et al. 1995; Schulze et al. 2001; Yan et al. 2002). *P* element insertions can be mapped to the polytenized domains of the chromocenter by in situ hybridization to salivary gland chromosomes (Devlin et al. 1990; Zhang and Spradling 1994; Roseman et al. 1995), but this approach does not provide significant information on the distribution of the insertions within the heterochromatin of diploid cells. Thus, high resolution FISH has been performed on neuroblast mitotic chromosomes of *D. melanogaster* (Zhang and Spradling 1994; Pimpinelli et al. 1995; Yan et al. 2002; Dimitri 2003), where heterochromatin can be subdivided into 61 cytologically distinct regions by banding techniques (Gatti et al. 1994). Here, we have performed FISH mapping of 13 *P* element insertions to the mitotic heterochromatin of chromosome 2 (Fig. 5, Table 4). These elements now assigned to specific bands represent useful landmarks for the physical mapping of heterochromatin (Yan et al. 2002; Dimitri 2003) and their remobilization should be useful for recovering insertional alleles or other derivatives. This approach might allow the identification of other 2Rh genes that are not yet characterized at the molecular level. For example, we have evidence suggesting that the remobilization of the 19.74.3 insert can induce novel lethal alleles of the *l(2)41Aa* gene (N. Corradini, F. Verni, F. Rossi and P. Dimitri, unpublished). Experiments are in progress to clarify the molecular nature of these mutations.

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