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DNA underreplication in intercalary heterochromatin regions in polytene chromosomes of *Drosophila melanogaster* correlates with the formation of partial chromosomal aberrations and ectopic pairing

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Abstract We studied the influence of the *Suppressor of Underreplication* (*SuUR*) gene expression on the intercalary heterochromatin (IH) regions of *Drosophila melanogaster* polytene chromosomes. We observed a strong positive correlation between increased *SuUR* expression, underreplication extent, amount of DNA truncation, and formation of ectopic contacts in IH regions. *SuUR* overexpression from heat shock-driven transgene results in the formation of partial chromosomal aberrations whose breakpoints map exclusively to the regions of intercalary and pericentric heterochromatin. It is important to note that all these effects are seen only if *SuUR* overexpression is induced during early stages of chromosome polytenization. Therefore, we developed the idea that ectopic pairing results from the joining of free DNA ends, which are formed as a consequence of underreplication.

Introduction

Polytene chromosomes of *Drosophila melanogaster* harbor two types of heterochromatin, namely, the classic pericentric heterochromatin and approximately 250 sites of intercalary heterochromatin (IH). The IH is represented by dense bands, which are scattered along the euchromatic arms of polytene chromosomes and share many common features with pericentric heterochromatin (reviewed by

Zhimulev 1998; Henderson 2004; Wallace and Orr-Weaver 2005). These attributes include transcriptional silencing and dense packaging, late DNA replication and underreplication, contacts with the nuclear lamina, association with silencing proteins, and frequent ectopic pairing. However, in contrast to the pericentric heterochromatin, IH regions are largely devoid of highly repeated sequences and, thus, should be considered as dispersed loci containing unique sequence DNA that are subject to silencing (reviewed by Zhimulev and Belyaeva 2003). Recent microarray analysis of sequences, which are underreplicated in salivary gland polytene chromosomes, clearly demonstrated that IH regions indeed comprise clusters of unique genes (6 to 40 genes each) whose replication and transcription profiles are coregulated (Belyakin et al. 2005). Still, the organization and functioning of IH regions remain poorly understood.

One of the most enigmatic features of IH regions is their ability to form ectopic contacts with each other and with pericentric heterochromatin. On chromosome squashes, ectopic contacts appear as intimate associations of IH bands or as structureless chromatin fibers between these bands. According to the in situ hybridization data, these fibers contain DNA molecules from bands that they join (reviewed in Zhimulev 1998). The phenomenon of ectopic pairing was first described in late 1930s (Bridges 1935; Kaufmann 1939; Prokofyeva-Belgovskaya and Khvostova 1939). Chromosome regions capable of forming ectopic contacts were subsequently mapped by Slizynski (1945), Kaufmann and Iddles (1963), and in more detail by Zhimulev et al. (1982).

At present, two main hypotheses for the nature of ectopic pairing are proposed. First, heterochromatic proteins, such as HP1, may be involved (Zhimulev 1998; Sage and Csink 2003). This is supported by the fact that increased amounts of HP1 bound by chromosomal regions result in higher frequency of their involvement in forming “ectopic loops,” presumably via changes in chromatin structure and silencing (Seum et al. 2001). An alternative hypothesis links ectopic contacts with DNA underreplication in heterochro-

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matin. It is believed that when replication forks stall, free, “sticky” DNA ends appear. These are subsequently ligated to form continuous molecules that join nonhomologous regions. This idea is based on the observed positive correlation between underreplication in IH regions and the frequency of ectopic contacts (Ashburner 1980; Zhimulev et al. 1982). Consistent with these data, truncated DNA molecules, which might have free double-stranded DNA ends, are found in the underreplicated heterochromatic regions (Leach et al. 2000).

The *Suppressor of Underreplication (SuUR)* gene is involved in the control of DNA underreplication in *D. melanogaster* salivary gland polytene chromosomes. The SUUR protein localizes to the IH and pericentric heterochromatin regions (Makunin et al. 2002). The *SuUR* mutation completely suppresses underreplication in the IH, partially in pericentric heterochromatin, and leads to the abolishment of ectopic pairing. Conversely, extra *SuUR* gene doses lead to the dramatic increase in the extent of underreplication and ectopic pairing (Belyaeva et al. 1998; Zhimulev et al. 2003).

We used the *SuUR* gene as a tool to investigate the relationship between different features of IH regions. Therefore, we examined whether SUUR directly influences ectopic pairing or if effects could be indirect and result from its influence on underreplication. In the present work, we report that *SuUR* overexpression leads to a higher frequency of ectopic pairing only when induced in embryogenesis and during the first two instar larval stages when most of the endocycles in salivary gland cells take place. When induced later in the third instar larvae, high amounts of the SUUR protein associating with the chromosomes fail to drive the formation of ectopic contacts. We also observed that increases in DNA underreplication in IH caused by extra doses of *SuUR* leads to massive truncation of DNA molecules in these regions and the formation of partial chromosomal aberrations.

Materials and methods

Fly stocks and constructs

All stocks and mutations used are described in FlyBase (Drysdales et al. 2005). The *SuUR* mutation was originally detected in the *In(1)sc^{V2}* stock due to its unusual effects on the phenotype of polytene chromosomes observed as a suppression of DNA underreplication in IH and pericentric heterochromatin. *SuUR* homozygotes are viable and fertile. The *SuUR* mutant stock contains a 6-kb insertion in the last exon of the gene (see Belyaeva et al. 1998; Makunin et al. 2002 for details). Flies were maintained at 18 or 25°C on standard cornmeal yeast-agar medium.

The *H7* construct contains *yellow⁺*, entire open reading frame and part of the 3'-untranslated region of the *SuUR* gene, which is under the control of the *hsp70* promoter. The *y w⁶⁷H7-X*, *H7-3* stock is homozygous for two insertion sites in the regions 4F and 91F and it therefore contains four copies of the *H7* transposon. Overexpression of the *hs-*

SuUR transposon rescues the phenotype of the *SuUR* mutant. The *miniwhite⁺*-marked X6S1 construct is composed of a 4.9-kb *XbaI-SalI* genomic fragment encompassing the functional *SuUR* gene (Makunin et al. 2002). The *y w⁶⁷* stock carrying two X6S1 transposons in addition to two doses of the endogenous *SuUR* is referred to as stock with four doses of the *SuUR* gene (4× *SuUR⁺*).

Pulsed-field electrophoresis and quantitative Southern blot hybridization

Nuclei were isolated from 100 salivary glands and 50 sets of larval brains and imaginal discs; treatment of DNA in agarose inserts was performed according to the standard protocol (Karpen and Spradling 1990). The DNA was digested with *NotI* and fractionated by pulsed-field electrophoresis on a BioRad DRII contour-clamped homogeneous electric field apparatus. Electrophoresis conditions were 6 V/cm, 5- to 50-s pulses at 14°C for 21.5 h in 0.5× Tris–borate–EDTA.

To obtain clones from the 11A region of chromosome X and the 75C region of chromosome 3, genomic DNA was amplified by polymerase chain reaction (PCR) using the following primers: 5'-ctgctgggacagcaaatc-3' and 5'-caaatcgtaacatggagac-3' (11A region, contig AE003487), 75CN+180 (5'-atcaaatccatctggcaacg-3' and 5'-taccgcactgaggcacaact-3', 2.3 kb), 75CN-187 (5'-gtaggtagtgtttgctt-3' and 5'-tactctcactcaccatc-3', 3.5 kb) (75C region, contigs AE003521 and AE003519, respectively). The PCR fragments were cloned into pBluescript II KS plasmid vector using appropriate restriction sites: 1.79-kb *PstI* fragment containing the *Upfl (CG1559)* gene from 11A region, 1.90-kb *SacI-XhoI* fragment from 75CN+180, and 3.50-kb purified PCR fragment 75CN-187 were used as hybridization probes for the analysis of the 11A and 75C regions.

After pulsed-field gel electrophoresis, DNA was transferred to the Hybond-NX (Amersham Biosciences) and analyzed by Southern blot hybridization as described earlier (Glaser and Spradling 1994). Probes were labeled with ³²P[dATP] to specific activities of approximately 10⁹ cpm/μg by random priming. Hybridization signals were analyzed qualitatively using Kodak XAR5 film.

For quantitative Southern blot hybridization, genomic DNA was isolated from 50 salivary glands and from 25 sets of larval brains and imaginal discs and cut with *HindIII* (MBI Fermentas). Digested DNA was size-separated on a 0.8% agarose Tris–acetate–EDTA gel and transferred to Hybond-NX (Amersham Biosciences) according to the manufacturer's specifications. The oligonucleotide primer pairs used and corresponding sizes for the region 11A were as follows: G0–50 (5'-catagcctttgccatct-3' and 5'-ttcactcattcccacc-3', 1.7 kb), G40–50 (5'-cacggcttaaccgcatta-3' and 5'-ctcgtcacaacgggact-3', 1.8 kb), G100–150 (5'-tcagcgcagttcacctt-3' and 5'-ggaccctgtttattatctttt-3', 1.5 kb), G150–200 (5'-ttgtgggtaaggattgt-3' and 5'-gtcattgcctattgtg-3', 2.0 kb), G200–250 (5'-gcgataagccgatcaaca-3' and 5'-agccacgggaagacaaag-3', 1.6 kb), G300–350 (5'-gacccgaagcatagag-3' and 5'-aagcccagactgtaacc-3', 1.6 kb), G350–

400 (5'-atacagcacaacatacgcacag-3' and 5'-ggcccttatgatgacgtactacc-3', 1.9 kb), and G400–450 (5'-attcactattgcccact-3' and 5'-attgcaagctcggttat-3', 1.7 kb). *EcoRI* DNA fragment (2.5 kb) from *Ten-A* gene cloned into pBluescript was kindly provided by E. Baumgartner. The oligonucleotide primer pairs used and corresponding sizes for the region 19E were as follows: R(-100) (5'-gtttcggcgtgaattct-3' and 5'-tcagtctgtcctgtgtt-3', 2.4 kb), R(-50) (5'-cttgactgcgcttccttc-3' and 5'-taacctatccacgaccttg-3', 1.4 kb), R(1–15) (5'-atcgacggagttcaatagc-3' and 5'-gtggaggagtgcaggaaa-3', 1.9 kb), R(30–40) (5'-tcgctgatggaggaggtgag-3' and 5'-gtcccgatgtgcgtgaagt-3', 1.4 kb), R(100–110) (5'-taagtgtacttcgggactgc-3' and 5'-ctgtgagggtaccatctg-3', 1.5 kb), R(136–140) (5'-actcaaaaccgaccacagc-3' and 5'-tgcgttctaccgccaga-3', 1.4 kb), R(230–240) (5'-agatttcag ttgctggataa-3' and 5'-atggaggacagtagtataacg-3', 1.2 kb), R(270–280) (5'-ctgtgcctctacatcttg-3' and 5'-agttctgccatcc ttctt-3', 2.2 kb), R(315–320) (5'-ttccatatttccattgctg-3' and 5'-atttatgtcgaagc ttgc-3', 2.4 kb), R(370–380) (5'-tattctaacgaggatcact-3' and 5'-tggcgtattctatacagag-3', 2.0 kb), R(417) (5'-tgaatcgaatcga atatctc-3' and 5'-tactctaaacttatgtaggc-3', 1.8 kb), and R(467) (5'-acggattagacgaatgaca-3' and 5'-accaagcactgaagcacc-3', 2.7 kb). Quantitative Southern-blot hybridization was carried out as described previously (Belyaeva et al. 1998). Relative DNA abundance was calculated as the ratio of hybridization signal in the salivary glands to that in diploid tissues after normalization with *rosy* signal.

Heat shock treatment

Eggs were collected for 2 h at 25°C, then at different time points, the cultures were heat shocked by immersing the vials in a 37°C water bath for 40 min. Embryos survive heat shock treatment after 3 h of development, so the earliest treatment possible with our cultures was 5 h after egg laying. We noted that daily heat shocks starting from 5–6 h of embryonic development up to the mid-third instar age result in a delay of larval developmental of about 2 days.

Cytology

Preparations of the salivary gland polytene chromosomes stained with acetic orcein were made by the standard method and analyzed by phase-contrast microscopy to determine the frequency of weak spots and ectopic contacts as described earlier (Zhimulev et al. 1982). The number of preparations and chromosomes examined is given in the “Results” section.

Results

Frequency of ectopic pairing is correlated with the extent of DNA underreplication

Local DNA underreplication in IH regions of polytene chromosomes is observed cytologically as chromosome breaks or “weak spots.” The *SuUR* mutation was

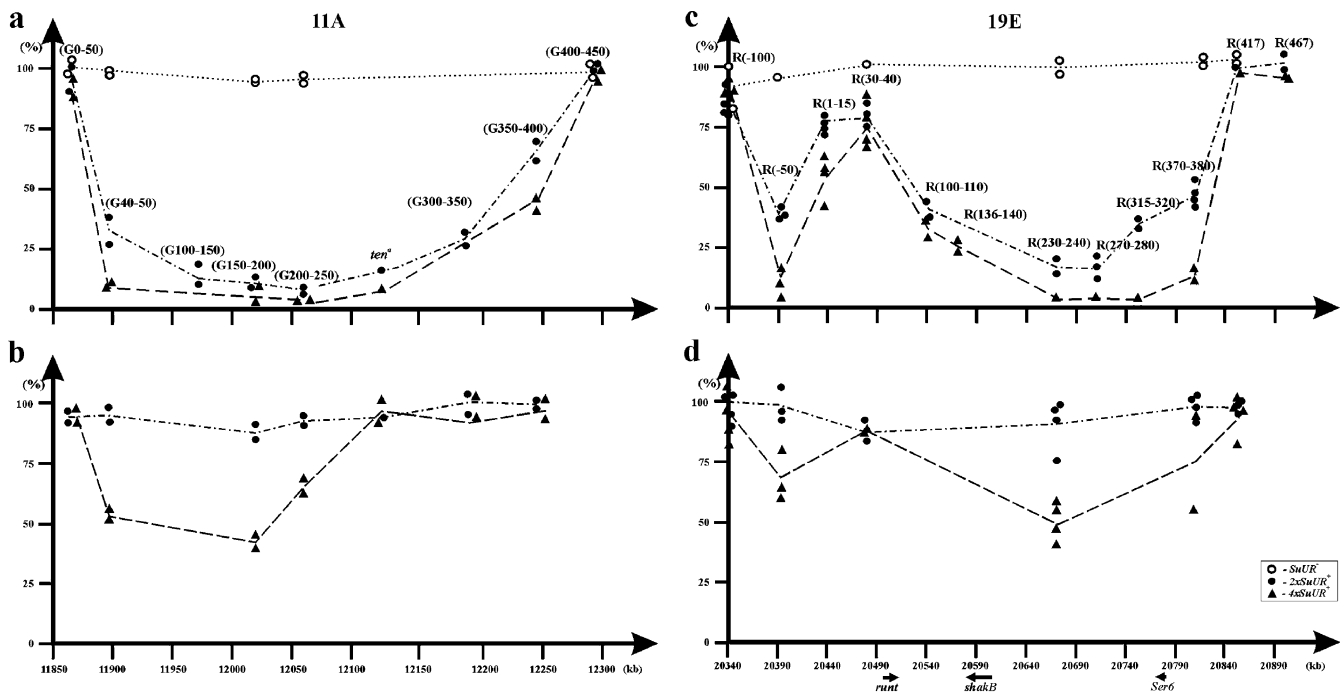


Fig. 1 Influence of *SuUR*⁺ extra doses on the polytenization profile in the regions 11A6–9 (a, b) and 19E (c, d) of the X chromosome in females (a, c) and males (b, d). *Abcissa*: distance in kilobase pairs according to *Drosophila* genome project r4.1. *Ordinate*: relative level of DNA replication (%) in the salivary gland cells, determined by Southern blot analysis

discovered due to its unique phenotype: suppression of the formation of weak spots. Additional copies of *SuUR*⁺ lead to the increased frequency of weak spots in regions that typically form breaks in the wild-type strain and cause the appearance of breaks at numerous novel sites where they are not normally observed. Influence of *SuUR* dosage on IH regions is most prominent for the male X chromosome. In wild-type stocks, this chromosome differs from the autosomes and the female X chromosomes in that it completes replication much earlier and shows no underreplication, weak spots, or ectopic contacts (see Zhimulev 1998 for review). In the stock that carries four copies of the *SuUR* gene, the male polytene X displays weak spots in IH (Zhimulev et al. 2003), which indicates these regions are underreplicated. To confirm this, we used Southern blot hybridization to analyze DNA abundance profiles in several IH regions of the X chromosome in males and females. Figure 1 illustrates the results obtained for the well-known IH regions 11A6–9. In the females of the wild type Oregon R stock, the region 11A harbors a 400-kb-long underreplication zone with the DNA amount in the central part of the zone present at 10% of level typical for

completely replicated regions (Fig. 1a). The degree of underreplication in the 11A region of female Oregon-R larvae, perhaps, reaches maximum because in the stock containing four doses of the *SuUR*⁺ gene, no further increase in underreplication is seen, neither in the degree of DNA representation (Fig. 1a) nor in frequencies of weak spots (Zhimulev et al. 2003). In the Oregon R male polytene X, region 11A is polytenized completely (Fig. 1b) and therefore lacks any visible breaks. Male X chromosomes in transgenic stock 4× *SuUR*⁺ stock display some degree of underreplication, although it is less pronounced than in females: (1) the underreplication region is narrower and (2) DNA representation level remains relatively high, approximately 50% in the central part of this region. Cytologically, this degree of underreplication results in infrequent chromosome breaks (about 10%) (Zhimulev et al. 2003). Similar results were obtained for yet another IH region on the X chromosome, namely, the region 19E (Fig. 1c,d). As a peculiarity of the 19E region, we could mention that this region contains two zones of underreplication. Both display lower profile of DNA representation in 4× *SuUR*⁺ than in Oregon R females (Fig. 1c).

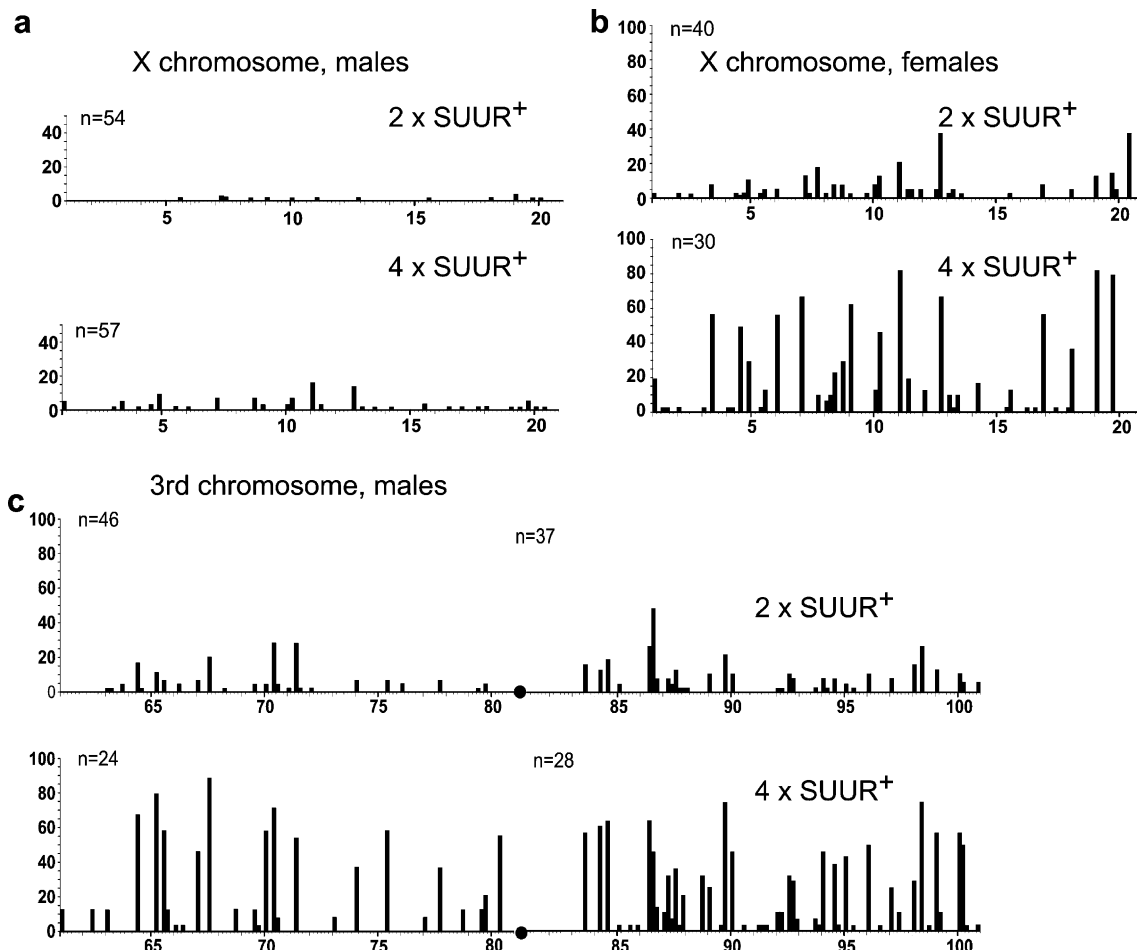


Fig. 2 Frequencies of ectopic contacts in polytene chromosomes from stocks bearing different doses of the *SuUR* gene. Chromosome regions are denoted on the *abscissa*; frequencies of the ectopic contacts formed by the region are plotted on the *ordinate* [ratio of

the number of chromosome regions participating in ectopic contacts to the number of regions observed (%)]. *n* means the number of chromosomes studied

Oregon R males do not demonstrate underreplication in this region, while in $4\times SuUR^+$ males, the underreplication is clear, however, it is not so profound as in females. Thus, $SuUR^+$ extra doses suppress the effects of dosage compensation on replication in males, which normally allows for the complete replication of all IH chromosome regions.

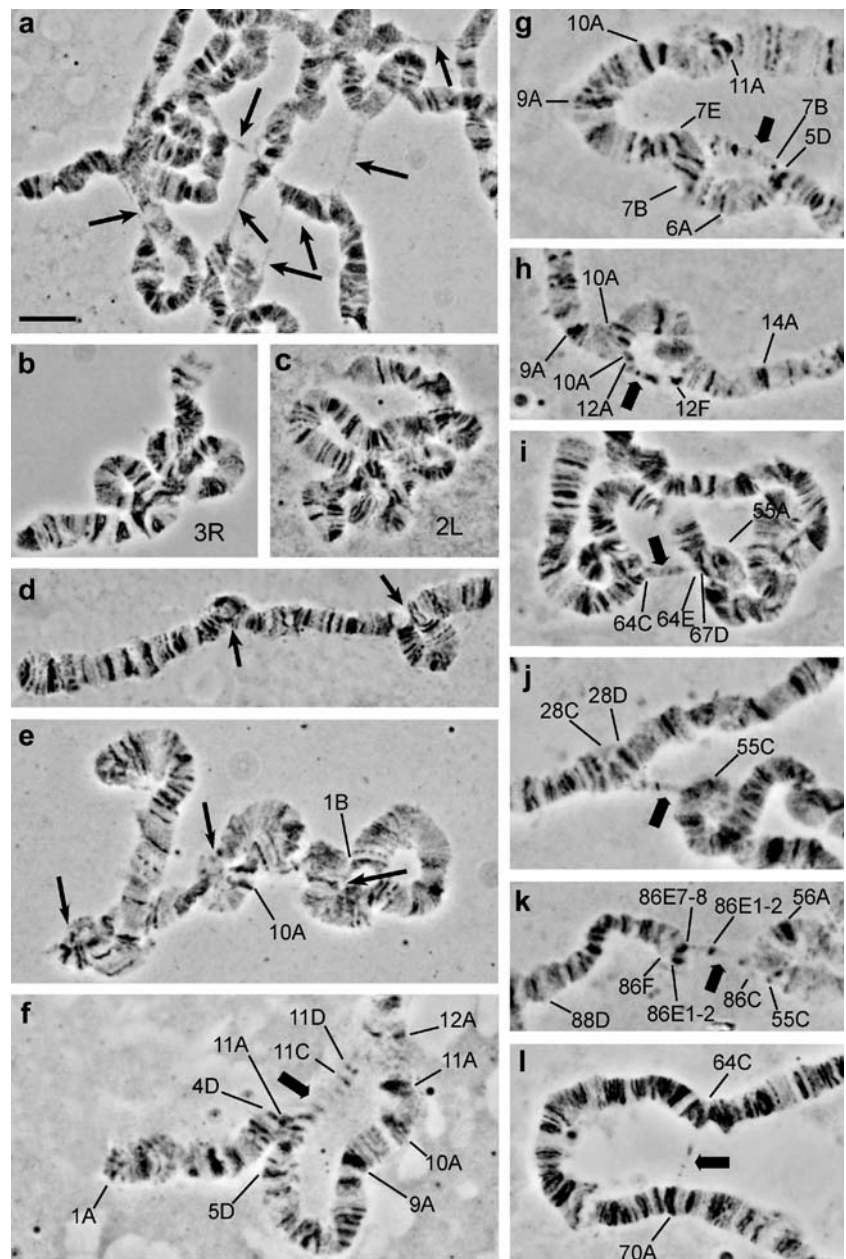
The appearance of underreplication is paralleled by ectopic contacts in the male X (Fig. 2a). In the case of X chromosomes of females and autosomes, additional $SuUR^+$ doses also cause significant increases in the frequency of ectopic contacts (Fig. 2b,c).

To summarize, the frequency of ectopic contacts strongly correlates with the replication extent, which is determined by the amount of SUUR protein.

Underreplication and ectopic pairing are induced only if $SuUR$ acts at early stages of chromosome polytenization

We then asked which developmental stages were important for SUUR to contribute to the formation of ectopic contacts. To answer this question, we took advantage of heat shock induction of ectopic $SuUR$ expression at different time points during development. We made use of a transgenic stock *H7*, which bears two insertions of a transposon containing the $SuUR^+$ placed under the heat shock promoter and is a homozygous mutant for the endogenous $SuUR$. We previously demonstrated that this system was functional: 2 h after the heat shock pulse in third instar larvae, the SUUR protein is readily detected on chromosomes. It is bound to most of the polytene

Fig. 3 Ectopic pairing and partial aberrations in chromosomes of the *H7* stock subjected to daily heat shocks. **a** General view of a polytene nucleus with numerous chromatin fibers joining the ectopically paired regions; **b**, **c** 3R and 2L chromosome arms display numerous knots of ectopic contacts; **d** singular “band-to-band” ectopic contacts in the male X chromosome; **e** multiple *knots* formed by ectopic contacts in the male X chromosome. **f–l** Examples of partial chromosome aberrations: **f–i** intra-chromosomal aberrations within single arm, **j** aberration between the 2L and the 2R chromosome arms, **k** aberration between the 2R and the 3R chromosome arms, and **l** thin branch observed in the chromosome of a wild-type stock without heat shock treatment. Regions are designated according to the Bridges polytene map (Bridges 1935); *thick arrows* point to branches, *thinner arrows* show ectopic contacts. *Bar* represents 5 μ m



chromosome bands (Zhimulev et al. 2003) with this localization pattern remaining unchanged even 16 h later (data not shown). Single heat shock in *H7* early embryos “rescues” the *SuUR*⁻ phenotype (Makunin et al. 2002).

After heat shock-driven *SuUR* overexpression beginning with early embryos of *H7* stock, female X chromosomes and autosomes display weak spots and ectopic contacts, which are so numerous that IH regions are often observed to be bundled into knots, which hinders detailed cytological analysis (Fig. 3a–c). Therefore, to quantify these changes, we used the male X chromosome where the weak spots are less pronounced. In the control, when rearing *H7* larvae at 18°C, the male polytene X lacks virtually any detectable breaks or ectopic contacts (Table 1, row 8), however, upon single heat shock treatment of 5- to 6-h-old *H7* embryos, about 50% of chromosomes show 1 to 2 ectopic contacts (Table 1 and Fig. 3d). Very similar results are seen when heat shocking 18–20 h *H7* embryos (Table 1, rows 2 and 3). The effect of applying a single heat shock is comparable to the effect of introducing two *SuUR*⁺ extra doses (Table 1, row 1).

When applying two heat shocks (5–6 and 18–20 h embryos), the percentage of chromosomes displaying singular ectopic contacts reaches 83% and chromosomes having multiple ectopic contacts (hence, impossible to count precisely) become frequent (Table 1, row 4 and Fig. 3e). Daily heat shocks from the onset of embryonic development up to the mid-third instars result in the strongest effect: more than half of all chromosomes display multiple ectopic contacts and the chromosomes that do not form an ectopic contact are very rare (Table 1, row 5). This pronounced effect of *SuUR* overexpression on ectopic pairing frequency is accompanied by stronger DNA underreplication, affecting now even the male polytene X. This is illustrated by higher frequencies of weak spots in

the IH regions. When analyzing 100 chromosomes from male larvae that were reared on a daily heat shock regime, the frequency of chromosome breaks in the region 3C reaches 40% and in the regions 7B, 11A, 12E, and 19E, it constitutes as much as 60–70%. These values are considerably higher than those observed in the 4× *SuUR*⁺ stock, having corresponding frequencies typically below 20% (Zhimulev et al. 2003). These data reinforce the link between the frequency of ectopic pairing and the amount of *SuUR* protein.

It is worth noting that the influence of *SuUR* on ectopic contacts is restricted to stages of early development: embryonic, first and second instar larval stages. Heat shock induction of the *SuUR* gene in third instar larvae (20 and 44 h before pupariation) does not result in any changes in chromosomes compared to the control (data for the female X and autosomes are not shown) (Table 1, rows 6 and 7). Accordingly, no changes in ectopic pairing were seen when overexpressing *SuUR* in mid-third instars via the *Sgs3-GAL4>UAS-SuUR*⁺ system (data not shown). Therefore, the increased amount of *SuUR* per se is not sufficient to induce the formation of ectopic contacts. The influence of *SuUR* on ectopic pairing is only seen when expressing *SuUR* at early stages of chromosome polytenization, therefore, *SuUR* appears to act through its effect on underreplication.

Frequency of ectopic pairing correlates with the extent of DNA truncation

As was shown earlier, underreplication of DNA in the regions of eu- to heterochromatin transition leads to the synthesis of truncated DNA molecules, their abundance being proportional to the underreplication degree (Glaser et

Table 1 Ectopic contacts in the male polytene X chromosome in the 4× *SuUR*⁺ stock and in the *H7* stock upon heat shock (HS) induced *SuUR* overexpression

Experimental conditions	Numbers of chromosomes studied	Frequencies of different chromosome types (%)		
		No ectopic contacts	1–2 ectopic contacts (see Fig. 3d)	Multiple (3 or more) ectopic contacts (see Fig. 3e)
1 4× <i>SuUR</i> ⁺ stock, all development at 18°C	100	45	54	1
2 HS of 5–6 h embryos ^a	86	48	52	0
3 HS of 18–20 h embryos	147	43	54	3
4 Double HS of embryos at 5–6 and 18–20 h	100	7	83	10
5 Daily HS beginning with 5–6 h embryos	100	1	38	61
6 HS of third instar larvae (44 h before fixation)	97	100	0	0
7 HS of third instar larvae (20 h before fixation)	90	98	2	0
8 Control, no HS (all development at 18°C)	102	98	2	0

^aHere and below the stock used was *H7*

al. 1997; Leach et al. 2000). The bowl-like profile of DNA representation, as observed in regions 11A and 19E (See Fig. 1), suggests that level of polytenization decreases from the edges inwards, reaching a value of 5 to 10% in the center of underreplication zone. This pattern also suggests that in IH regions, replication forks stall on many strands of the polytene chromosomes, which result in truncated DNA molecules. Direct evidence that this is indeed the case comes from pulsed-field gel electrophoresis data, allowing a direct estimate of the length of DNA molecules in IH regions (Fig. 4).

The DNA in the region 11A of wild-type males, which have two *SuUR*⁺ doses is represented by a full-length 456-kb-long *NotI* fragment, having only trace amounts of truncated DNA molecules. This observation corresponds well with the absence of underreplication in this region, which was shown using quantitative Southern-blot hybrid-

ization (see Fig. 1). When isolated from salivary glands of males having four *SuUR*⁺ doses, the 11A DNA appeared as a pool of severely truncated DNA fragments. The truncated DNAs were heterogeneous in size, ranging from approximately 100 kb up to the size of the 456 kb full-length restriction fragments; molecules in the range of 170 to 310 kb were the most prominent. In *H7* larvae, the number of truncated molecules increases progressively with stronger *SuUR* overexpression induced by heat shock. The truncated fragments are practically absent from the larvae reared at 25°C; they become numerous after double heat shock treatment at embryonic and first instar larval stages and are most abundant in larvae that were subjected to daily heat shocks throughout development. The increase in the number of truncated molecules is accompanied by the reduction in the amount of the full-length fragment. In general, the same is applicable to the autosomal region

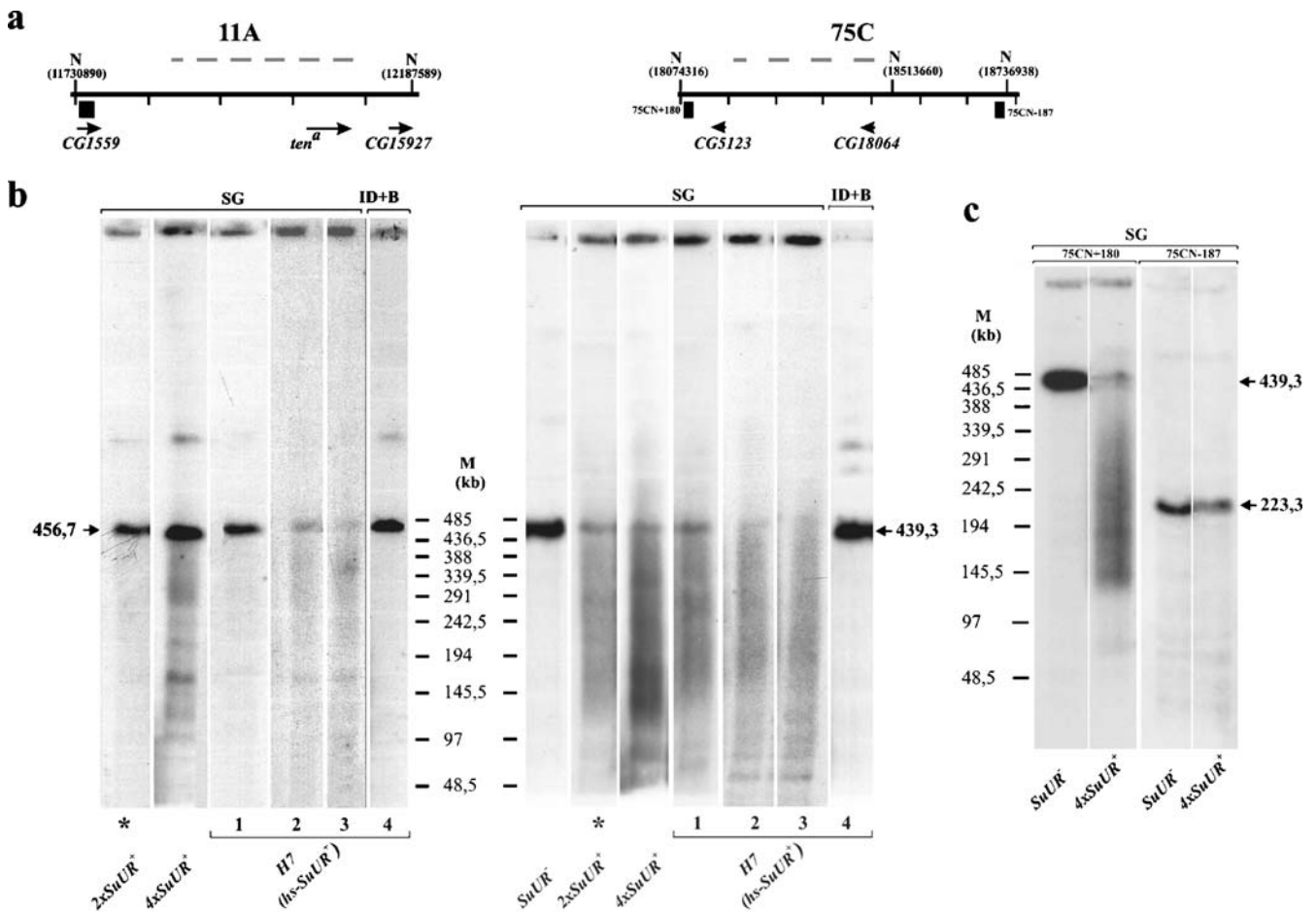


Fig. 4 Truncation of DNA molecules in the IH regions 11A and 75C (in males). **a** Physical DNA maps of the IH regions 11A and 75C within *NotI* restriction fragments (N) are shown (solid lines) in a 100-kb scale with positions determined according to *Drosophila* genome project, r 4.1 (<http://www.flybase.org>). Approximate localization of underreplication zones (dashed gray lines) in salivary glands of third-instar larvae (4× *SuUR*⁺) is presented according to data for male 11A (Fig. 1b) and Belyakin et al. (2005) for 75C. Black squares below the maps correspond to DNA fragments used as the probes for Southern blot hybridization; selected transcription units (horizontal arrows) are given as

reference points. **b, c** Separation of DNAs using pulsed field gel electrophoresis followed by Southern blot hybridization. SG Salivary glands and ID+B imaginal discs and brains. Genotypes and experimental conditions are given below the lanes: asterisks Oregon R, daily heat shocks; lanes designated with 1–4 mean experiments with *H7* strain: 1 no heat shock, development at 25°C; 2 two heat shocks, in embryos and first instar larvae; 3 daily heat shocks starting with early embryos; and 4 double heat shock during the third instar stage (36 and 20 h before dissection). Lambda phage DNA concatemers were used as a molecular mass ruler (M)

75C, except for the fact that as was expected, in a wild-type stock with $2\times SuUR^+$ doses, the truncated DNA molecules and the DNA underreplication are readily detectable. It is also apparent in the *H7* stock without heat shock, which can be attributed to the leaky nature of the heat shock promoter. Truncation degree appears higher upon stronger *SuUR* expression ($4\times SuUR^+$ and *H7* after heat shock) so that in the daily heat shocked *H7* larvae, there can be practically no full-length fragment detected. Truncated DNA molecules are absent in the region 75 only in the *SuUR*⁻ background (Fig. 4).

It must be noted that the correlation between the amount of underreplicated truncated molecules and the extent of *SuUR* overexpression is observed only in the salivary gland polytene chromosomes, but not in the diploid tissues of imaginal discs and brains. In these tissues, the truncated DNA molecules were not detected even if the *H7* larvae were daily heat shocked throughout the third instar larval stage (Fig. 4). It is important to note that the heat shock treatment alone does not alter the size of the DNA fragments because the truncated DNA molecules in the region 11A are not observed in wild type Oregon R stock ($2\times SuUR^+$) when isolated from the salivary glands of male larvae reared with daily heat shocks (Fig. 4).

To control for the DNA integrity, we compared hybridization patterns for two different probes on the same filters (Fig. 4c). Two left lanes on the figure demonstrate results of hybridization with 75CN+180 probe: absence of truncation for the *SuUR*⁻ strain and strong truncation for the $4\times SuUR^+$. Then the same filters were reused for hybridization with 75CN-187 probe, which is localized on the proximal part of the 229.3-kb *NotI* fragment. It is situated beyond the underreplication zone of the 75C IH region and so it is completely represented in polytene chromosomes (Fig. 4a). In this case, the only signal is visible; it corresponds to a full-length 223.3-kb-long fragment. The picture of hybridization does not depend on the dosage of the *SuUR* gene (Fig. 4c, two right lanes). These data show that genomic DNA is intact outside the zones of underreplication, therefore, short DNA fragments in the IH region are not artifacts; DNA truncation, we observed, is a consequence of underreplication in IH.

To summarize, we observe a strong correlation between the strength of *SuUR* expression, the frequency of breaks, ectopic pairing, and the amount of truncated DNA molecules in the IH of polytene chromosomes.

Overexpression of *SuUR* leads to formation of partial chromosome aberrations

The majority of the numerous ectopic contacts observed upon *SuUR* overexpression display morphology, which is indistinguishable from the one that is typical for the wild-type chromosomes. Most often, these contacts can be described as associations between the IH bands that have the material of one band continued in another band with the chromosomes being bent in the sites of contact (Fig. 3).

Apparently, due to the stretching of ectopically paired bands, the unstructured bundles joining the IH regions are formed (Fig. 3a). The characteristic feature of ectopic contacts upon *SuUR* overexpression is that many IH regions tend to associate as complex knots (Fig. 3b,c).

Besides higher frequency of these typical ectopic contacts, *SuUR* overexpression results in a distinct morphological phenotype, which involves the generation of partial chromosomal aberrations when only some chromonemes of the polytene chromosome appear rearranged (Fig. 3f-k). The rearranged subchromosomal material becomes visible only when it is separated from the chromosome body and forms a "branch." Figure 3f exemplifies a situation when a partial aberration occurred between regions 11A and 4D. As a result, the rearranged bundle of chromatids lacking a vast region from 4D to 11A is substantially shorter than its counterpart in the chromosome body. Obviously, because they are stretched, these rearranged strands are found to be partially detached from the common bundle of conjugating chromonemes and thus form a branch between 4D and 12A. In regions other than 4D and 12A, the conjugation of chromonemes in a rearranged material of the chromosome remains unaffected. Thus, branches appear as a morphological marker of a partial aberration, which forms in postmitotic nuclei of salivary gland cells.

The branches could also be viewed as a consequence of asynapsis of homologs. However, this is not the case because they are always thinner than one of the unpaired homologs (their width being no greater than a quarter of the diameter of a chromosome) and also because they are observed to form both in autosomes and in a single male X chromosome. Having analyzed about 100 salivary gland chromosome squashes, we found 70 cases with branches showing distinct banding pattern; however, we were able to reliably map the breakpoints in only 26 of them. We believe the number of branches is underestimated because upon *SuUR* overexpression, chromosome analysis becomes very difficult due to numerous ectopic contacts and chromosome breaks. Also, many branches, which were too thin to be examined, could still be seen in as many as 30% of nuclei. Partial aberrations were mainly found in chromosome squashes from larvae having *SuUR* overexpressed very early in development. The earlier induction is, the thicker branches are, obviously because the key events for generation of partial aberrations, i.e., chromosome breaks and end joining occur during the first endocycles, afterwards the rearranged chromatids undergo additional rounds of polytenization so that branches become visible under the light microscope. The branches were observed to vary in diameter, which supports the idea that they were generated at different time points of polytene chromosome formation.

In wild-type chromosomes, we failed to detect branches that would be as thick as 1/8 to 1/16 of the chromosome diameter, as was regularly observed on *SuUR* overproduction. The faint branches above the light microscope resolution threshold were very rare in the wild-type polytene chromosomes (Fig. 3l).

Branches often do not display clear banding pattern because they are usually very stretched and are quite thin. When possible, breakpoints of a partial aberration were mapped. As it is shown in Table 2, in most cases the aberrations affected one chromosome arm. In many cases, vast material up to half a chromosome arm long (such as 4D-11A), is lost, however, more frequently the deletions cover smaller neighboring regions. Based on the morphology, genuine inversions and deficiencies are difficult to be discerned (see “Discussion”).

In all cases studied so far, the joining of breakpoints occurred in heterochromatic regions and most often, these were the regions of the IH (complete list of the IH regions is given in Zhimulev et al. 2003). It is interesting to note that the asynapsis points of branches with the rest of the chromatids of a polytene chromosome tend to map to the IH regions as well. This is apparently because of stronger synapsis of chromonemes in the IH regions (Zhimulev et al. 1982).

The data obtained suggest that *SuUR* overexpression leads to stronger underreplication in the IH regions, thereby increasing the amount of truncated DNA molecules and making ectopic contacts more frequent. In addition, partial chromosomal aberrations appear. Furthermore, these phenotypes are a specific effect of *SuUR* dosage.

Table 2 Mapping the partial chromosome aberrations arising in polytene chromosomes of the third instar larvae after daily heat shock beginning with 5–6 h embryos

Chromosomes affected	Junction sites (breakpoints)	End positions of branches
X	5D–7B	7E
X	1B–4D	1C
X	10A–13A	9E; 13E
X	8E–11A	11C
X	7B–11D	12A
X	4D–11A	12A
X	1E–3A	1D
X	10A–12A	12F
X	3C–4AB	4E1-2
X	7B–6A	5D
X	7B–8AB	6D
X-3R	10B–86D	86E
2L	24D–34A	22A-B
2L	26C–29DE	26C
2L	36D–37A	38A
2L-2R	28D–55A?	28C; 55C?
2L-chromocenter	36C-chromocenter	36D
2R	59D–57A	60E
2R-3R	55C–86C	86E7-8
3L	62E–64D	62D
3L	64E–67D	64C
3L	71C–67A	70F
3R	85A–86D	87AB
3R	88A–87D	88C
3R	94D–96A	94A
3R	98C–95A	94D

Overexpression of a number of heterochromatic proteins, such as HP1, SU(VAR)3–7, and PC does not result in higher frequency of weak spots, ectopic contacts, or formation of partial chromosome aberrations (data not shown).

Discussion

The results presented corroborate previous data (reviewed in Zhimulev 1998) on the correlation of underreplication extent and the frequency of ectopic contacts. Therefore, these data clearly implicate SUUR in affecting both of these phenomena. We further established that the binding of the SUUR protein to the chromosomes per se does not result in stronger underreplication or in ectopic pairing. These phenotypes are only seen upon early SUUR expression in embryos and first- and second-instar larvae, i.e., during the first endocycles in the salivary glands (Orr-Weaver 1994). The data obtained argue that the earlier underreplication is induced, the stronger it is manifested in polytene chromosomes of third instar larvae because the underreplicated DNA molecules are “amplified” in subsequent endocycles. Also, novel underreplicated chromatids are generated with each cycle, so the number of underreplicated molecules increases.

In the present work, we develop the idea that the ectopic contacts and fibers observed on polytene chromosome squashes result from joining the truncated molecules, which are formed in the IH regions due to the underreplication. Cytological evidence of this joining of IH regions follows from the observation of partial chromosomal aberrations induced by *SuUR* overexpression.

Similar branches (partial aberrations) were originally found in polytene chromosomes of *Drosophila* (Slizynski 1950) after irradiation of early embryos. Then they were characterized in detail in *Drosophila* and *Chironomus* after irradiation (Hilliker 1985; Keyl 1958) and after treatment of embryos and early larvae with FudR (Hägele 1971). Keyl (1958) defined “partial mutation (rearrangement)” as a recombination of breaks involving only a group of chromatids of one of the polytene chromosome homologs occurring exclusively in postmitotic chromosomes. Partial aberrations described in our paper are morphologically similar to the aberrations that arise upon irradiation of embryos and early larvae. They affect only some chromatids of polytene chromosome and display peculiar branches or “side connections” as originally termed by Slizynski (1950). The side connections result from local asynapsis of the rearranged subchromosomal bundle with the rest of the strands in polytene chromosome. The width of a rearranged bundle of chromatids depends on the induction time: the earlier, the thicker. Finally, the chromosomes at late polytenization cycles (in the third instar larvae) are no longer sensitive to the *SuUR*-driven induction, i.e., no aberrations are observed.

In both X-ray treatment and *SuUR* overexpression systems, the ratio of intra- to interchromosomal aberrations is very similar. However, in contrast to the radiation-

induced aberrations, which involve, according to Hilliker (1985), not only the IH regions (55% of the breakpoints found in the work of Hilliker fall into the IH), all the aberrations identified in our experiments always had breakpoints mapping to the underreplicated IH regions (Table 2). There was only one case of a breakpoint found in pericentric heterochromatin. We suggest that in our case the partial aberrations are based on the phenomenon of DNA underreplication. It is worth noting that Hägele (1971) drew similar conclusions based on the induction of partial chromosome aberrations by treating *Chironomus* embryos and early larvae with FUdR, an agent known to block DNA replication by inhibiting thymidine synthesis.

The formation of chromosomal aberrations is always accompanied by the generation of free ends (breaks) in DNA followed by their joining. The existence of free double-stranded ends in DNA molecules that were subject to underreplication during endocycles was demonstrated in the work of Leach et al. (2000) who studied replication in the transition region between eu- and heterochromatin in polytene cells of *D. melanogaster*. These authors suggested that “non-homologous end-joining reaction between free-ended DNAs at different loci would produce ectopic ligations between the regions that were underreplicated.” Polytene chromosomes undergo endocycles despite incomplete DNA replication (Lilly and Spradling 1996) and, thus, might accumulate DNA molecules with free double-stranded ends (Leach et al. 2000). Furthermore, evidence demonstrating that discontinuous DNA molecules are formed in underreplicated IH regions follows from the DNA representation profile (Fig. 1). In the middle part of the underreplication region over 90% of the DNA strands are underrepresented; therefore, given that the underreplicated zone spans hundreds of kilobase pairs, the sequences between the replication-stalled ends of truncated molecules appear deleted. Other regions of IH (52 regions were found in microarray studies by Belyakin et al. 2005) show a similar picture of underreplication in the salivary gland polytene chromosomes. Further accumulation of truncated DNA molecules in IH is directly demonstrated by pulsed-field gel electrophoresis (see Fig. 4). Additional doses and overexpression of *SuUR*⁺ result in production of more truncated DNA molecules and conversely, in the *SuUR* mutants these regions are completely replicated. It remains to be established what the exact mechanisms of *SuUR* influence on replication are: whether *SuUR* influences chromatin compaction thereby compromising replication fork progression or acts directly on the replication machinery. Whatever were the cases, upon *SuUR* overexpression, more underreplicated truncated DNA molecules appear.

In agreement with Leach et al. (2000), we assume that the free DNA ends that arise because of underreplication can join together by DNA repair mechanisms, which will result in the formation of continuous rearranged DNA molecules. This conclusion further extends the ideas on the contribution of underreplication to the formation of ectopic

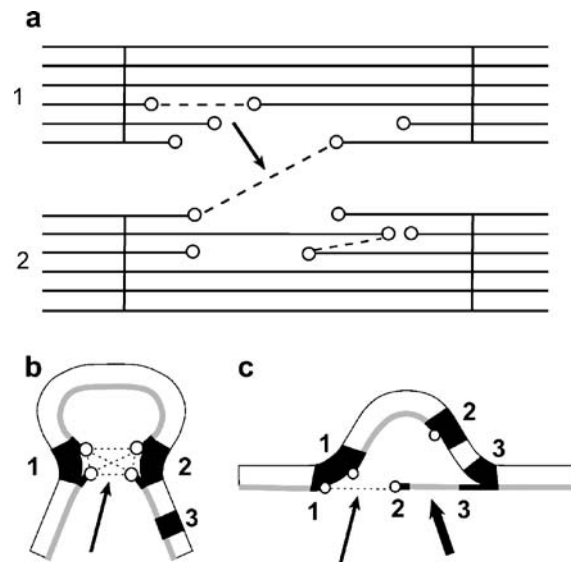


Fig. 5 Hypothetic scheme of formation of partial chromosomal aberrations. **a** Two nonhomologous IH regions (1 and 2) of a polytene chromosome are shown. Horizontal lines represent chromonemes and vertical lines delimit underreplication zone within IH regions. Some DNA strands are continuous, i.e., fully replicated, some are underreplicated and therefore have their ends free (circles). Joining the free DNA ends within a single IH region results in the formation of a continuous thread bearing a microaberration (deletion and duplication) whose exact nature depends on which free ends were ligated (dashed lines). Ligation of two free ends from different chromosome regions may result in the formation of the ectopic thread (thin arrow). **b, c** explanation of branch formation. Ligation of the free DNA ends from different regions will result in a chimeric molecule that will join these regions: **b** the “band-to-band” (1 to 2) ectopic pairing or **c** the branch will be observed. **b** Polytene chromosome (white) has one of its chromonemes underreplicated (gray) with free DNA ends (white circles) in two IH regions (black) that are found close to each other. Underreplicated chromoneme fully conjugates with the completely replicated homologous threads of a polytene chromosome. Each of the underreplicated molecules has two free ends. Ligation (thin arrow) of four free DNA ends available in these two regions occurs in random combinations (dashed lines) and, thus, would produce a rearranged chromoneme that would subsequently replicate. This chromatin thread would fix the “band-to-band” contact. **c** Situation essentially analogous to that shown in subpanel **b** except for the loss of synopsis of a rearranged thread with the homologous region in the chromosome body (thick arrow) with the formation of deficiency. Branch formed between the junction point and one of the neighboring IH regions (marked as “3”).

contacts. These concepts were formulated in the “replication fork transfer” hypothesis (Ashburner 1980) and in the hypothesis of the generation of “sticky” ends in IH (Zhimulev et al. 1982). The formation of partial chromosomal aberrations, which accompanies stronger underreplication upon *SuUR* overexpression established in the present work, demonstrates cytological evidence for these hypotheses because breakpoints of these aberrations always locate in the underreplicated IH regions.

The same events, i.e., formation of breakpoints and their joining, apparently underlie all types of ectopic contacts occurring between different regions of IH and pericentric heterochromatin (Fig. 5). If the junction of two breakpoints occurs within one underreplication zone, continuous

molecule bearing a microdeletion or a microduplication or other type of aberration is formed, remaining virtually undetectable by conventional cytology. If free DNA ends from different regions are joined, the continuous thread of ectopic contact appears (Fig. 5a). Thus, ligation of free double-stranded DNA ends, which occurs in the underreplication zone is always accompanied by a partial aberration; however, the resulting morphology may be different depending on whether the synapsis of the rearranged strand with the chromosome body is disturbed or not (Fig. 5b,c).

We can detect the aberrations by cytological means in the cases where partial aberrations are formed as a result of polytenization of the rearranged strands in the course of subsequent endocycles when these strands are found in partial asynapsis with the chromosome body (Fig. 5c). If this does not occur, the typical ectopic “band-to-band” pairing is observed or the unstructured chromatin bundle containing the material of one or both contacting bands emerges (Fig. 5b).

Why are there no additional ectopic contacts formed upon *SuUR* overexpression in the third instar larvae? This is possibly attributable to the intensity of the generation of free DNA ends. Leach et al. (2000) suggested that the free double-stranded DNA ends are amplified in subsequent replication cycles after replication fork stalls. We hypothesize that in the third instar larval chromosomes, which undergo final replication rounds, the underreplicated molecules having their ends free are relatively too few on the one hand, and on the other hand, they are more limited in time to be ligated and amplified.

Figure 5 illustrates the hypothesized scheme of the aberrations generated due to underreplication when the ends of only one chromosome strand are joined. However, taking into account numerous underreplicated strands locating in the IH regions and the complex ectopic contacts of several distinct chromosome regions forming knots, one can imagine more complex aberrations (Keyl 1958). It should be noted that it is not the exact type of aberration that is important for the purposes of this work, but the very occurrence of the aberration because it confirms the possibility of recombination between the IH regions.

Joining the free DNA ends in the context of chromatin requires that they are found in close proximity in the nucleus before the first endocycles. Most probably, such a compartment for the IH regions to merge is nuclear lamina in the interphase of the last mitotic cycle before the onset of polytenization. The association of IH and pericentric heterochromatin with the nuclear lamina of the polytene nucleus in third instar larvae was reported earlier (Hochstrasser and Sedat 1987). It was also shown that in the interphase nucleus the polytene chromosome arms occupy distinct nuclear regions. Based on the distribution of chromosome junctions in partial chromosomal aberrations obtained by radiation, Hilliker (1985) concluded that in early embryonic nuclei of *Drosophila*, each of the interphase chromosomes occupies a relatively specific domain of the nucleus with significant folding within the chromosomal arms. Thus, one can imagine the chromo-

some as fixed on the membrane via its heterochromatic regions with the intervening regions looping out. Association of IH regions can be also related with compartmentalization of Pc-G target genes (Bantignies et al. 2003 and references therein); about 70% of the IH regions bind Pc-G protein antibodies as it was shown by Zhimulev et al. (2003).

Underreplication appears crucial for the ectopic pairing to occur; however, we believe it is likely that some other silencer proteins, such as HP1 and Pc-G, could contribute to this phenomenon. Ectopic contacts were observed to be formed at increased frequencies upon local tethering of HP1 to the euchromatic sites (Seum et al. 2001; Li et al. 2003), which was accompanied by silencing of a reporter gene. Perhaps, in some cases, overexpression of chromatin proteins induces nonspecific “sticking” of neighboring chromosome regions, which are best seen after applying formaldehyde-containing fixatives that cross-link the protein and DNA molecules. In particular, such a situation was described in the work of Li et al. (2003) whereby the ectopic threads were DNA-negative. So, the stickings observed have apparently nothing in common with the chimeric molecules formed within “genuine” ectopic contacts. Alternatively, we cannot eliminate the suggestion that these artificially constructed IH regions, binding HP1, were also binding SUUR, i.e., the HP1-induced effect described was indirect. This possibility requires further experimental evidence. We nevertheless believe that many heterochromatic proteins and their interactions might play important roles in the spatial organization of cell nucleus. They could contribute to the bringing of chromosomal regions into proximity, mediate their contacts with nuclear lamina, or promote telomeric associations.

It is also important to note that the associations of the chromosome loci seen in diploid cells are not equivalent to the ectopic pairing observed in polytene chromosomes. Ectopic pairing is a consequence of underreplication, a phenomenon characteristic only for the polytene tissue.

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