The Heterochromatic *rolled* Gene of *Drosophila melanogaster* Is Extensively Polytenized and Transcriptionally Active in the Salivary Gland Chromocenter

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ABSTRACT

This paper reports a cytogenetic and molecular study of the structural and functional organization of the *Drosophila melanogaster* chromocenter. The relations between mitotic (constitutive) heterochromatin and α - and β -heterochromatin are not fully understood. In the present work, we have studied the polytenization of the *rolled* (*rl*) locus, a 100-kb genomic region that maps to the proximal heterochromatin of chromosome 2 and has been previously thought to contribute to α -heterochromatin. We show that *rolled* undergoes polytenization in salivary gland chromosomes to a degree comparable to that of euchromatic genes, despite its deep heterochromatic location. In contrast, both the *Bari-1* sequences and the AAGAC satellite repeats, located respectively to the left and right of *rl*, are severely underrepresented and thus both appear to be α -heterochromatic. In addition, we found that *rl* is transcribed in polytene tissues. Together, the results reported here indicate that functional sequences located within the proximal constitutive heterochromatin can undergo polytenization, contributing to the formation of β -heterochromatin. The implications of this finding to chromocenter structure are discussed.

CONSTITUTIVE heterochromatin is an almost ubiquitous component of chromosomes of higher organisms. While euchromatin condenses and decondenses at specific phases of the cell cycle, constitutive heterochromatin retains a compact structure throughout most of the cell cycle (HEITZ 1928). Despite extensive data on heterochromatin, its biological significance and the reason for its widespread occurrence are still largely unknown (reviewed by JOHN 1988). However, genetic, cytological and molecular studies have shown that *Drosophila melanogaster* constitutive heterochromatin, which comprises ~30% of the entire genome, is a functional biological material and contains several active genes (reviewed by GATTI and PIMPINELLI 1992; WEILER and WAKIMOTO 1995; ZUCKERKANDL and HENNING 1995).

D. melanogaster mitotic (constitutive) heterochromatin contains large blocks of highly repeated satellite DNAs (APPELS and PEACOCK 1978; BONACCORSI and LOHE 1991; ABAD *et al.* 1992; LOHE *et al.* 1993). It also harbors a number of middle repetitive DNAs, some of which belong to different transposable element families (CARLSON and BRUTLAG 1978; DI FRANCO *et al.* 1989; SHEVELYOV *et al.* 1989; TRAVERSE and PARDUE 1989; CAIZZI *et al.* 1992; PIMPINELLI *et al.* 1995). In *D. melanogaster* polytene tissues, the heterochromatic regions from all chromosomes aggregate to form a cytological structure called the chromocenter. Two cytological do-

main components of heterochromatin can be distinguished in the chromocenter (HEITZ 1934): α -heterochromatin, which corresponds to a small compact region located in the middle of the chromocenter and undergoes little if any replication during polytenization (GALL *et al.* 1971), and β -heterochromatin, a diffusely banded mesh-like material that lies between euchromatin and α -heterochromatin. The α -heterochromatin is known to contain underrepresented satellite DNA sequences (GALL *et al.* 1971), while the β -heterochromatin of all chromosomes appears to be enriched in transposable elements (YOUNG *et al.* 1983; ANANIEV *et al.* 1984; MIKLOS *et al.* 1988; VAURY *et al.* 1989; DEVLIN *et al.* 1990). Although β -heterochromatin is clearly polytenized (GALL *et al.* 1971; LAKHOTIA 1974), its replication degree relative to that of both euchromatin and α -heterochromatin has only been ascertained for two β -heterochromatic genes, *i.e.*, *suppressor of forked*, located at the euchromatin-heterochromatin junction on the X chromosome, and *light*, which maps to the distal mitotic heterochromatin of 2L; in both cases the degree of polytenization approaches 80% of that of euchromatin (DEVLIN *et al.* 1990; YAMAMOTO *et al.* 1990).

The assembly and organization of the chromocenter represents one of the most intriguing aspects in the mitotic to polytene chromosome transition. The relationship between constitutive heterochromatin of mitotic chromosomes and the α - and β -heterochromatin of polytene chromosomes has been analyzed in several studies. It has been generally thought that the bulk of constitutive heterochromatin forms the α -heterochromatin, while the chromosomal regions bordering con-

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stitutive heterochromatin contribute to the β -heterochromatin (HEITZ 1934; LAKHOTIA and JACOB 1974; YAMAMOTO *et al.* 1990; reviewed by MIKLOS and COTSELL 1990). However, this model rests upon the assumption that the location of α - and β -heterochromatic domains within the chromocenter actually reflects that of mitotic chromosomes. In *D. melanogaster* three lines of evidence are not completely consistent with this conclusion. First, in polytene nuclei the rDNA genes, which are located within the constitutive heterochromatin, attain an intermediate degree of replication relative to those of euchromatin and satellite DNAs (SPEAR and GALL 1973) and are sequestered from the chromocenter into the nucleolus (PARDUE *et al.* 1970; reviewed by SPRADLING and ORR-WEAVER 1987). Second, TRAVERSE and PARDUE (1989) found that the heterochromatic copies of HeT sequences, which are not underrepresented in polytene chromosomes (YOUNG *et al.* 1983), colocalize with satellite DNAs; they proposed that while satellite DNA aggregates into the α -heterochromatin during chromocenter formation, the HeT DNA sequences are looped out to form β -heterochromatin. Finally, PZ transposons inserted in autosomal heterochromatin have been found to be fully represented in salivary gland DNA, despite their proximal location relative to satellite DNA blocks (ZHANG and SPRADLING 1995). These studies together indicate that specific classes of sequences mapping to the constitutive heterochromatin have the ability of undergoing polytenization and therefore escape the classification proposed by MIKLOS and COTSELL (1990).

A further contribution to the understanding of chromocenter organization might come from the study of single-copy genes that are naturally located within the proximal heterochromatin. High-resolution cytogenetics techniques coupled with molecular analysis can be employed to assess whether single-copy genes mapping to the deep mitotic heterochromatin escape polytenization. One such heterochromatic gene is *rolled*, a mitogen-activated protein kinase-encoding gene (BIGGS *et al.* 1994; BRUNNER *et al.* 1994) that is essential for both viability and for cell proliferation during imaginal disc development (HILLIKER 1976; DIMITRI 1991). The *rolled* gene is one of the most proximally located heterochromatic genes of 2R and maps to region *h40-h41*, close to the centromeric region *h38* (DIMITRI 1991). Therefore, unlike *suppressor of forked* or *light*, *rolled* is clearly located within a pericentromeric region expected to form α -heterochromatin according to the model proposed by MIKLOS and COTSELL (1990).

In the present study, we have investigated the degree of replication of *rolled* in salivary gland nuclei by both fluorescence *in situ* hybridization (FISH) and Southern blot assays. In parallel, polytenization studies were also carried out using the single-copy euchromatic glutamine synthetase 1 (*GS1*) gene (CAIZZI *et al.* 1990), the *Bari-1* middle repetitive element (CAIZZI *et al.* 1992) and the (AAGAC)_n satellite DNA repeats (LOHE *et al.* 1993).

In addition, we have studied *rolled* gene transcription in both polytene and diploid tissues. The results of these analyses show that *rolled* is transcriptionally active and fully replicated in polytene nuclei despite its deep heterochromatic location.

MATERIALS AND METHODS

Fly stocks: Genetic markers, mutations and special chromosomes used in this work were described by LINDSLEY and ZIMM (1992). The second chromosome inversion *In(2Rh)PL41A-B;53C5-9*, whose mitotic heterochromatic breakpoint maps to *h39* proximal to the *Bari-1* cluster, was induced by X-ray treatment on a chromosome 2 carrying a *P[w⁺]* insertion in region *53C5-9* of the polytene chromosome map (L. PASSERI and M. GATTI, personal communication). This inversion places the *P[w⁺]* element close to the centromeric region *h38* and was selected because of its strong *white-variegated* eye phenotype. Fly stocks were maintained on standard *Drosophila* medium at 25° (+/-) 1°.

Chromosome preparations, fluorescent *in situ* hybridization and charge-coupled-device (CCD) camera analysis: Mitotic chromosome preparations from larval neuroblasts were prepared as described by PIMPINELLI and DIMITRI (1989). Larval salivary glands were dissected in saline solution (0.7% NaCl) and squashed in 45% acetic acid. DNA probes were routinely labeled by nick translation using biotin-11-dUTP (Enzo), and signals were detected with fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). FISH and CCD camera analysis were carried out as described in detail elsewhere (GATTI *et al.* 1994). For colocalization experiments, fluorescent hybridization signals were obtained by simultaneously using a biotinylated probe that was detected by FITC-conjugated avidin and a second probe labeled with digoxigenin and detected by a rhodamine-conjugated antibody. Both salivary gland and mitotic chromosome preparations were stained with 4',6-diamidino-2-phenylindole (DAPI). Images were merged and analyzed by using the Adobe Photoshop 2.5 program. The *Bari-1* and *r1* DNA probes used in FISH experiments are described below; the AAGAC repeats were detected using probe 198 (BONACCORSI and LOHE 1991; LOHE *et al.* 1993).

DNA hybridization probes: The *rolled* cDNA subclone was kindly provided by E. HAFEN and L. ZYPURSKY. The hybridization probe was a 1.4-kb cDNA *Hind* III restriction fragment containing the complete coding region of the gene (BIGGS *et al.* 1994). As a control for single-copy gene mapping to the second chromosome euchromatin, we used a *Eco*RI genomic fragment from the glutamine synthetase (*GS1*) gene (CAIZZI *et al.* 1990). We also used a 1.7-kb *Bari-1* *Hind*III restriction fragment (CAIZZI *et al.* 1992) as a middle repetitive DNA control.

DNA preparation and Southern blot hybridization assays: Larval salivary glands and brains from third instar larvae of both Canton-S and Oregon-R strains were dissected in 0.7% NaCl and were ground with a pestle in an Eppendorf microfuge tube in lysis buffer (30 mM Tris-HCl pH 8, 100 mM EDTA). Proteinase K was added to 0.1 mg/ml and Sarkosyl to 1%. After incubation at 42° for 2 hr, the mixture was deproteinized by extraction with phenol, phenol/chloroform (1:1) and chloroform. The DNA was precipitated with ethanol, recovered by centrifugation and resuspended in 10 mM Tris-HCl (pH 8) and 1 mM EDTA. DNA electrophoresis and transfer onto nylon membranes (Hybond-N, Amersham), probe radiolabeling with α -³²P, filter hybridization and washing were carried out following standard procedures (SAMBROOK *et al.* 1989). DNA molecular weight markers were *Bst*EII restriction fragments of λ DNA.

FIGURE 1.—FISH mapping of *rolled*, *Bari-1* and AAGAC DNA sequences on mitotic heterochromatin of chromosome 2. (A) DAPI staining of Canton-S second chromosomes pseudocolored in blue. (B) Merging of fluorescent *rolled* hybridization signal pseudocolored in red. The *rolled* signal maps to the distal end of region *h41*; the *rolled* was labeled with biotin and detected by FITC-conjugated avidin. (C) DAPI staining of Canton-S second chromosomes pseudocolored in blue. (D) Merging of the *in situ* hybridization signal from *Bari-1* se-

FIGURE 2.—Cytological map of chromosome 2 heterochromatin showing the localization of *rolled*, *Bari-1* and AAGAC sequences. Filled areas represent the Hoechst 33258-bright regions, the shaded boxes represent regions of intermediate fluorescence and the open boxes are regions of dull fluorescence. The *Bari-1* cluster maps to *h39*, the *rolled* locus maps to the distal part of region *h41*, and the AAGAC satellite DNA maps to two sites: a prominent signal maps to *h43*, while a minor signal is localized to the proximal end of region *h46*.

RNA preparations and Northern blot hybridization assays:

Total RNA was isolated from 40-2nd instar larvae, 100 brains and 100 pairs of salivary glands. Fat bodies for total RNA extraction were collected from 100 third instar larvae. Samples were transferred in 70 μ l extraction buffer (20 mM NaCl, 20 mM Tris-HCl pH 8, 40 mM EDTA, 1% SDS plus 20 μ g of yeast tRNA), frozen in liquid nitrogen and homogenized with a glass rod. Total RNA was extracted three times with phenol/chloroform and once with chloroform. Purified RNA was precipitated according to CROWLEY *et al.* (1984) and kept at -20° overnight. The pellet was resuspended in 30 μ l of pyrocarbonic acid diethyl ester-treated H_2O . The RNA was fractionated by electrophoresis through 1% agarose gels containing 5% formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham) as described in SAMBROOK *et al.* (1989). RNA filters were prehybridized and hybridized for 16 hr at 42° in 50% formamide, $5\times$ SSC, $5\times$ Denharts' solution, 0.5% SDS, 10 mM EDTA (pH 8) and 100 μ g/ml ssDNA and washed at 65° in $0.5\times$ SSC, 0.1% SDS. Probe labeling with α - ^{32}P was carried out following standard procedures (SAMBROOK *et al.* 1989).

RESULTS

FISH mapping of *rolled*, *Bari-1* and AAGAC satellite DNA repeats to the 2R heterochromatin: We initially undertook a detailed FISH mapping of *rolled*, *Bari-1* and AAGAC satellite sequences. The results of this analysis are shown in Figure 1 and diagrammatically summarized in Figure 2.

The *rolled* locus was previously mapped to the very proximal *h40-h41* region of the 2R heterochromatin

quences pseudocolored in yellow. The *Bari-1* probe was labeled with digoxigenin and detected by a rhodamine-conjugated antibody. *Bari-1* DNA maps to region *h39*. (E) DAPI staining of Oregon-R second chromosomes pseudocolored in blue. (F) Merging of both *rolled* and *Bari-1* hybridization signals; colors as above. Note that the *rolled* signal is distal to that of *Bari-1*. (G) DAPI staining of Canton-S second chromosomes pseudocolored in blue. (H) Merging of AAGAC hybridization signals pseudocolored in purple. The AAGAC probe was labeled with digoxigenin and detected by a rhodamine-conjugated antibody. One prominent AAGAC signal extends through region *h43* to the proximal portion of region *h44*, while a signal of weaker intensity localizes to the proximal end of region *h46*. (I) DAPI staining of *In(2Rh)PL, 41A-B; 53C5-9* pseudocolored in blue; merging of the *in situ* hybridization signal from *Bari-1* sequences pseudocolored in yellow (right). The heterochromatic breakpoint of this inversion maps to region *h39*, proximal to *Bari-1* cluster, since it displaces the *Bari-1* signal from the centromere.

(DIMITRI 1991). To obtain a higher resolution mapping of *rolled* within 2R constitutive heterochromatin, we used the 1.4-kb *rolled* cDNA probe (BIGGS *et al.* 1994) in FISH experiments to mitotic chromosomes of either Canton-S or Oregon-R strains. Simultaneous FISH experiments were also carried out with both *rolled* cDNA and *Bari-1* probes. These experiments (Figure 1, A and B) enabled us to further restrict the localization of *rolled* to the distal part of region *h41*. Because *rolled* is a single-copy gene (BIGGS *et al.* 1994), the *rolled* signal is faint; yet it is clearly distinguishable in ~30% of the prometaphase figures that were analyzed. *In situ* hybridization with *Bari-1* probe confirmed that this element is clustered into region *h39* (Figure 1, C and D). Simultaneous FISH experiments with both probes (Figure 1, E and F) clearly show that *rolled* maps to *h41*, distal to the *Bari-1* signal in *h39*. The fluorescence intensity of the *rolled* signal is reduced as compared to that produced by *Bari-1*, consistent with the finding that almost 80 copies of tandemly arranged *Bari-1* elements are clustered within region *h39* (CAZZI *et al.* 1993). Figure 1, G and H, shows that the localization of the AAGAC satellite DNA repeats is slightly different from that previously reported (LOHE *et al.* 1993): the AAGAC repeats map to two different sites within the 2Rh; one prominent signal extends through region *h43* to the proximal portion of region *h44*, while a signal of weaker intensity localizes to the proximal end of region *h46*.

FISH mapping of *rolled*, *Bari-1* and AAGAC satellite DNA repeats within the chromocenter of salivary gland chromosomes: FISH experiments with 1.4-kb *rolled* probe to salivary gland polytene chromosomes of either Oregon-R or Canton-S strains showed that the *rolled* signal lies within the inner regions of the chromocenter (Figure 3A). No other signals were seen at any other chromosomal location, indicating that no cross-hybridization occurred between the *rolled* cDNA probe and other related kinase domain-containing sequences. The *rolled* hybridization signal exhibits a strong fluorescence intensity, resulting in a large diffuse structure very different from the sharp hybridization signals usually seen with euchromatic probes. Interestingly, in some chromocenters *rolled* produces two distinct signals (Figure 3A), a pattern that suggests that sister chromatids retain their identity within the chromocenter. A similar pattern was produced by some *PZ* heterochromatic insertions (ZHANG and SPRADLING 1995).

The strong fluorescence intensity of the *rolled* signal (Figure 3A) suggests that *rolled* DNA underwent replication during polytenization to some extent. To further pursue this question, comparative FISH experiments with *rolled* and *Bari-1* sequences were carried out on the same salivary gland nuclei preparations (Figure 3, B and C). The analysis performed on several digital images showed that the *rolled* signal is eightfold stronger than that of the *Bari-1* heterochromatic cluster (Table 1); the latter often appears as a small dot, fivefold reduced

relative to the *Bari-1* euchromatic signals that were assumed to correspond to single-copy insertions. The *in situ* hybridization pattern of the AAGAC satellite DNA repeats within the chromocenter is shown in Figure 3D. Nearly 1800 kb of AAGAC sequences have been estimated to be present in the 2Rh heterochromatin of mitotic chromosomes (LOHE *et al.* 1993). However, underrepresentation of the AAGAC sequences in salivary gland nuclei results in a signal comparable to that of the single-copy *rolled* gene. Together, these results indicate that, unlike the *Bari-1* cluster or the AAGAC satellite DNA repeats, *rolled* undergoes extensive replication during polytenization. Thus, cytologically, the *rolled* gene, which maps to the proximal mitotic heterochromatin, appears to contribute to the polytenized portion of the chromocenter.

A more detailed mapping of *rolled*, *Bari-1* and AAGAC repeats within the 2Rh was performed on polytene chromosomes of larvae homozygous for the *In(2Rh)PL41A-B;53C5-9* inversion (Figure 3, E–G). This paracentric inversion was chosen because it displaces the bulk of 2R heterochromatin from the chromocenter into the 2R euchromatin. Polytene chromosome cytology shows that the proximal and distal breakpoints of this inversion are located in the proximal end of region *41* and in the euchromatic region *53C5-9*, respectively. The heterochromatic breakpoint within the 2R mitotic heterochromatin maps to *h39*, proximal to the *Bari-1* cluster that is completely displaced from the centromere (Figure 2, I and L). As shown in Figure 3, F and G, the signal distribution and intensity in salivary gland nuclei from larvae homozygous for *In(2Rh)PL* further support the conclusion that *rolled*, unlike *Bari-1* or the AAGAC repeats, identifies an extensively polytenized proximal region within the 2R heterochromatin.

Southern blot analysis of the *rolled* and *Bari-1* sequences in salivary gland and brain DNA: To further extend our cytological observations on *rolled* polytenization, we next determined the level of representation of *rolled* DNA in polytene compared to diploid tissues. The entire 1.4-kb *rolled* cDNA sequence was used to probe *Bam*HI-digested genomic DNA extracted from larval brains, salivary glands and whole adults. As a loading control, filters were simultaneously probed using a 400-bp genomic fragment from the glutamine synthetase 1 (*GSI*) gene, a single-copy euchromatic sequence from region 21 of 2L. The filters were also reprobated with a 1.7-kb *Bari-1* probe. The results of these experiments are shown in Figure 4. Southern blot analysis with the *rolled* cDNA probe identified nine genomic fragments (1–9). Although a detailed exon/intron map of the *rolled* locus is not available as yet, the pattern of hybridization shown in Figure 4A is consistent with the size of the genomic locus, which has been estimated to extend over 100 kb (BIGGS *et al.* 1994). The intensity of the *rolled* signal was normalized relative to that produced by the euchromatic *GSI* probe by microdensitometric

FIGURE 3.—FISH mapping of the *rolled*, *Bari-1* and AAGAC sequences within the chromocenter of salivary gland nuclei. (A) Localization of the *rolled* gene DNA within the chromocenter. The DAPI staining of Canton-S salivary gland chromosomes is pseudocolored in blue and merged to the *rolled* hybridization signal pseudocolored in red. The *rl* signal forms a large structure within the chromocenter and is split into two substructures. (B) Simultaneous *in situ* hybridization with *rl* and *Bari-1* DNA on Oregon-R salivary gland chromosomes. The *rl* (red) and *Bari-1* (yellow) hybridization signals are merged with DAPI staining (blue) of salivary gland chromosomes. (C) Simultaneous *in situ* hybridization with *rolled* and *Bari-1* DNA probes on Canton-S salivary gland chromosomes. The hybridization pattern is identical to that observed in the Oregon-R chromocenter. (D) *In situ* hybridization with the AAGAC satellite DNA probe (purple) on Canton-S salivary gland chromosomes. (E) Phase contrast picture of salivary gland chromosomes from larvae homozygous for *In(2R)PL* showing the displacement of almost all heterochromatin of 2Rh. Chr, chromocenter; 2Rh, heterochromatin of the right arm of chromosome 2 displaced from the chromocenter; 2R, right arm of chromosome 2. (F) Simultaneous *in situ* hybridization with *rolled* (red) and *Bari-1* (yellow) DNA probes on salivary gland chromosomes from larvae homozygous for *In(2Rh)PL*. (G) *In situ* hybridization with the AAGAC sequences (purple) on salivary gland chromosomes from females homozygous for *In(2Rh)PL*.

TABLE 1

rolled and *Bari-1* hybridization signal intensities in salivary gland chromosomes

Samples	<i>rolled</i>	<i>Bari-1</i> het	<i>Bari-1</i> eu
1	19500	2736	16082 17052 14160 19000
2	20340	2052	15600 10428 8748
3	20769	3717	14250 13000 13630 15000
4	31815	3331	15120
Mean ^a	23106 ± 5828	2959 ± 726	14339 ± 1592
Signal ratio			
<i>rolled</i> / <i>Bari-1</i> het			7.8
<i>rolled</i> / <i>Bari-1</i> eu			1.6
<i>Bari-1</i> eu/ <i>Bari-1</i> het			4.8

The signal intensity was quantified in four polytene nuclei from two Oregon-R larvae. Within the same cell single-copy *Bari-1* signals were observed in euchromatin in addition to the *Bari-1* and *rolled* heterochromatic signals (Figure 3). The mean value obtained for the *rolled* signal was compared to those of *Bari-1* heterochromatic and *Bari-1* euchromatic signals to estimate the relative intensity.

^a Values are ± 2 SE.

scanning of several independent autoradiographs. Six *rolled* fragments (2, 3, 4, 5, 7 and 8) exhibited a high level of representation ranging from 90% up to 100% in salivary gland DNA compared to brain DNA. The actual values were as follows: 95% for fragment 2, 90% for fragment 3, 98% for fragments 4 and 7, and 100% for fragment 8. A lower level of representation was observed for fragments 1 (67%) and 6 (77%). Reprobing the filters with the 1.7-kb *Bari-1* fragment (Figure 4B) showed that *Bari-1* DNA is clearly underrepresented in salivary gland compared to brain DNA (80-fold reduction), with the exception of two equally distributed fragments of 14 and 10 kb, which may correspond to *Bari-1* elements inserted into euchromatic sites. Together, these results clearly indicate that the *rolled* gene, despite its deep 2Rh location between underreplicated DNA blocks, undergoes extensive polytenization in salivary glands.

Northern blot analysis of the *rolled* transcript: Since the *rolled* gene is fully polytenized in salivary glands, we asked whether it is transcriptionally active in this tissue. We investigated *rolled* transcription in total larvae, salivary glands, fat bodies and brains. To control RNA loading, we used a DNA probe corresponding to the ribosomal protein 49 gene (*rp49*). These experiments revealed *r*-homologous transcripts in salivary glands and in whole second instar larvae (Figure 5), as well as

FIGURE 4.—Replication of the *rolled* gene DNA in salivary gland nuclei. Southern blot hybridization assays of Canton-S genomic DNA (1 µg) from whole adult flies, larval brains and salivary glands digested with *Bam*HI. Molecular weight marker sizes (λ *Bst*IE fragments) are indicated. (A) The whole 1.4-kb *Hind*III *rolled* cDNA probe, and the 0.4-kb *Eco*RI genomic fragment from the 3' end of the *GSI* gene were simultaneously used to probe the filter-bound DNA. Use of the *GSI* probe revealed that loading of genomic DNA from adult flies is slightly more abundant than that from brains and salivary glands. Fragment 7 required a long exposure to be satisfactorily identified, especially in the DNA from brains and salivary glands, which both were somewhat less abundant than that from adult flies as revealed by the *GSI* loading control (a). (B) The same filter was stripped and reprobed with the 1.7-kb *Bari-1* probe. The heterochromatic cluster of *Bari-1* (in the high molecular weight portion of the gel) is clearly underrepresented in salivary gland DNA.

in larval brains and fat bodies (data not shown). In particular, two transcripts of 2.6 and 1.9 kb were detected using the 1.4-kb *rolled* cDNA probe (Figure 5); both transcripts were absent in larvae homozygous for *Df(2Rh)Rsp1*, a heterochromatic deletion that removed, besides the *Rsp* locus, *rolled* as the only vital gene (GANETZKY 1977; DIMITRI 1991). These results indicate that both transcripts specifically result from the activity of the *rolled* gene. The larger 2.6-kb transcript was also detected in polyA⁺ RNA purified from young adults using the same *rolled* cDNA probe (P. C. R. EMTAGE and A. J. HILLIKER, personal communication). The 1.9-kb transcript may represent a *rolled* larval-specific transcript and was not identified in previous studies.

DISCUSSION

It has long been thought that the bulk of constitutive (mitotic) heterochromatin corresponds to the underreplicated α-heterochromatic regions of polytene chro-

FIGURE 5.—Northern blot analysis of the RNAs from the *rolled* gene. (A) An autoradiographic exposure of a Northern blot containing total RNA from second instar larvae (1) and salivary gland (3) of Canton-S strain and from second instar larvae homozygous for *Df(2Rh)Rsp¹* (2) that lack the *rolled* gene. The 1.4-kb *rolled* cDNA probe reveals two major transcripts of 2.6 and 1.9 kb, both absent in larvae homozygous for *Df(2Rh)Rsp¹*. The same filter was reprobed with the *rp49* ribosomal protein gene probe (O'CONNELL and ROSBASH 1984) as a loading control.

mosomes, while euchromatic-like segments bordering the constitutive heterochromatin would contribute to β -heterochromatin (reviewed by MIKLOS and COTSELL 1990). This model essentially rests upon genetic and molecular studies of the *X*-chromosome β -heterochromatin (SCHALET and LEFEVRE 1976; YAMAMOTO *et al.* 1990). In the present work, we have studied a *D. melanogaster* pericentric region lying within the proximal heterochromatin of 2R.

The *rolled* gene undergoes extensive polytenization in salivary glands despite its proximal location within the constitutive heterochromatin of 2R: By both cytological and molecular analysis, we have shown that the *rolled* gene, despite its proximal location in the constitutive heterochromatin (region *h41*), undergoes extensive DNA replication in salivary gland nuclei, in contrast to the severely underrepresented *Bari-1* cluster and AAGAC satellite DNA repeats that derive from different subdomains of 2Rh.

The present study reports the first example of a single-copy gene mapping to the proximal mitotic heterochromatin whose replication degree during polytenization is comparable to that of euchromatic sequences. The bands identified by Southern blot analysis are likely to include sequences flanking the *rolled* region, estimated to extend over 100 kb (BIGGS *et al.* 1994), as well as intronic parts of the gene; thus, it appears that polytenization extends throughout a relatively large genomic area. However, Southern analysis with the *rolled* cDNA probe revealed minor, yet consistently reproducible, variations (from 70 to 100%) among different genomic fragments composing the *rolled* locus, suggesting that the level of polyteny may not be homogeneous throughout the locus. This result may reflect a slight differential replication either within the *rolled* region or in the segments flanking the gene. A similar situation has been described for the *bithorax* complex, in which

a gradient of polyteny was observed within the transcription unit in salivary gland nuclei, such that the 5' end is fourfold underreplicated while the 3' end of the region is fully polytenized (LAMB and LAIRD 1987). Differential levels of representation were also seen within the polytenized array of rRNA genes, with 28S genes containing large underrepresented insertions (DECICCO and GLOVER 1983).

The fluorescent signal given by the *rolled* probe on salivary gland chromosomes produces a diffuse staining that extends over a relatively large area of the chromocenter, in agreement with the cytological pattern observed for the *light* gene (DEVLIN *et al.* 1990) and for certain heterochromatic, highly polytenized, *PZ* insertions (ZHANG and SPRADLING 1995). Thus, *rolled* DNA appears to form a large structure within the chromocenter, which may reflect the organization of genetically active heterochromatic sequences. This view is supported by our finding that the *rolled* gene is transcriptionally active in salivary glands.

The results of our analysis are in agreement with those obtained with the HeT-A sequences (TRAVERSE and PARDUE 1989) and with different *PZ* insertions (ZHANG and SPRADLING 1995), yet conflict with the classical notion that the proximal mitotic heterochromatin forms the underreplicated α -heterochromatin of polytene chromosomes and does not contribute to the formation of β -heterochromatin. This is particularly evident when examining the differential polytenization of the three heterochromatic elements *Bari-1*, *rolled* and the AAGAC satellite DNA repeats, all lying within the proximal 2R constitutive heterochromatin. The *Bari-1* cluster and the AAGAC sequences map to region *h39* (Figure 2) and to regions *h43-h44* and *h46*, respectively (Figure 2), and are both underrepresented in polytene chromosomes. In contrast, the *rolled* gene is extensively polytenized, despite its mapping to *h41*, proximal to the AAGAC sequences and to other characterized underrepresented satellite DNA blocks (LOHE *et al.* 1993). Thus, the level of polyteny of heterochromatic DNAs does not appear to be related to their position within the constitutive heterochromatin of 2R. Furthermore, it does not seem to be related to their sequence, since, for example, the *Bari-1* euchromatic copies, which are structurally similar to those mapping to *h39* (CAIZZI *et al.* 1993), undergo polytenization (Figures 3B and 4B). Our observation that *rolled* is transcriptionally active in salivary glands, as are other sequences contributing to the β -heterochromatin (BIESSMANN *et al.* 1981; MIKLOS *et al.* 1984; DEVLIN *et al.* 1990), suggests that the structural and/or functional state of specific heterochromatic domains plays an important role in determining their replication fate during polytenization. For example, it might be expected that during polytenization replication origins are accessible in transcriptionally active heterochromatin. Together, these findings lead us to conclude that the β -heterochromatin of 2R results

FIGURE 6.—Model for the formation of the chromocenter. During polytenization, portions of mitotic (constitutive) heterochromatin (indicated with B), containing transcriptionally active sequences, might remain accessible to the replication machinery; these regions, physically separated by underrepresented blocks (A), aggregate to form the β -heterochromatin. The remaining underreplicated DNA (A) constitutes the canonical α -heterochromatin.

from the aggregation of replicated domains of constitutive heterochromatin (Figure 6), possibly identifying transcriptionally active regions, physically separated by underreplicated areas. According to this view, multiple junctions between polytenized and nonpolytenized domains might exist within the chromocenter. Underrepresented α -heterochromatin within the chromocenter has been thought to be almost exclusively composed of satellite DNAs. However, the finding that the *Bari-1* cluster is severely underrepresented in salivary glands (Figure 4B) indicates that certain transposon-like heterochromatic sequences also fail to undergo polytenization.

Regions of complex genetically active DNA within the proximal heterochromatin of chromosome 2: We have shown that *rolled* maps to *h41*, a moderately Hoechst-fluorescent region of 2R mitotic heterochromatin. Other EMS-mutable genes were also mapped to the constitutive heterochromatin of chromosome 2 and showed an overall cytogenetic organization similar to that of *rolled* (DIMITRI 1991). *In situ* hybridization experiments revealed that these gene-containing heterochromatic regions from chromosome 2 harbor several clusters of sequences homologous to retroelements, such as *I*, *F*, *Doc*, *mdg-1*, *copia* and *gypsy* (PIMPINELLI *et al.* 1995), while lacking highly repetitive satellite DNAs (LOHE *et al.* 1993). This cytological organization is consistent with the molecular finding that both *light* (DEVILIN *et al.* 1990) and *suppressor of forked* (MITCHELSON *et al.* 1993) are surrounded by middle repetitive DNAs. These observations, together with the evidence that heterochromatic retroelements are transcribed in different *D. melanogaster* tissues (BUSSEAU *et al.* 1994), suggest that multiple domains of functionally active, complex DNA sequences, composed by single-copy genes and middle-repetitive elements, are present within the proximal

heterochromatin of chromosome 2. We believe that this cytogenetic organization reflects the underlying molecular structure of the *D. melanogaster* centric heterochromatin proposed by LE *et al.* (1995), where blocks or islands of complex DNA alternate with stretches of highly repeated satellite sequences.

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