Note

Chromosome Condensation Defects in *barren* RNA-Interfered Drosophila Cells

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ABSTRACT

Barren, the Drosophila homolog of XCAP-H, is one of three non-SMC subunits of condensin, a conserved 13S multiprotein complex required for chromosome condensation. Mutations in *barren* (*barr*) were originally shown to affect sister-chromatid separation during mitosis 16 of the Drosophila embryo, whereas condensation defects were not detected. In contrast, mutations in yeast homologs of *barren* result in defective mitotic chromosome condensation as well as irregular chromatid separation. We have used double-stranded RNA-mediated interference (RNAi) to deplete Barren in Drosophila S2 cells. Our analyses indicate that inactivation of *barr* leads to extensive chromosome condensation and disrupts chromatid segregation.

CHROMOSOME condensation in eukaryotic cells requires condensin, a 13S multiprotein complex that is conserved in all organisms studied to date (Hirano *et al.* 1997; Sutani *et al.* 1999; Freeman *et al.* 2000; Kimura *et al.* 2001). This complex, identified initially in Xenopus egg extracts, consists of five subunits termed XCAPs (Xenopus chromosome-associated proteins): two of these belong to the structural maintenance of chromosome (SMC) family and contain coiled-coils with globular terminal domains that interact with ATP and DNA (Kimura *et al.* 1999); the remaining three are defined as non-SMC proteins and play essential, although not well understood, roles in condensation (Kimura *et al.* 2001). Mitotic kinases control both localization of the condensin complex to chromosomes and its supercoiling and condensation activities (Kimura *et al.* 1998, 2001; Sutani *et al.* 1999; Giet and Glover 2001).

Two sets of evidence indicate 13S condensin is required for chromosome condensation. Immunodepletion of affinity-purified condensin fractions in Xenopus egg extracts converted compact sperm chromatin into a round-shaped interphase-like chromatin sphere, whereas adding condensin back restored the activity of chromosome assembly (Hirano *et al.* 1997). In yeast, mutations in the condensin subunits resulted in increased distance between two labeled probes on a mitotic chromosome and disorganization of rDNA array (Strunnikov *et al.* 1995; Ouspenski *et al.* 2000; Lavoie *et al.* 2002). This approach, coupled with biochemical analyses, has been exploited to define roles played by the SMC subunits in chromosome condensation and segregation, including the yeast homologs of the SMC-type subunit XCAP-C (Guacci *et al.* 1993; Saka *et al.* 1994; Strunnikov *et al.* 1995). More recently, mutants in the Drosophila SMC4 ortholog have been shown to be defective in proper chromosome condensation and segregation throughout different developmental stages (Steffensen *et al.* 2001).

Studies of the non-SMC subunits of condensin have been carried out mainly on Barren, the homolog of the XCAP-H subunit. *barren* (*barr*) was originally isolated as a Drosophila mutant affecting embryonic neuronal cell divisions (Bhat *et al.* 1996). The analysis of dividing cells in mutant *barr* embryos revealed that chromosomes fail to separate their chromatids during anaphase, giving rise to extensive chromatin bridging (Bhat *et al.* 1996). This phenotype is similar to that observed in DNA topoisomerase II mutants in *Saccharomyces cerevisiae* (Holm *et al.* 1989). *barr* chromosomes, however, appear to condense regularly and the missegregation phenotype does not appear to be a consequence of defects in chromosome condensation. Thus, the involvement of *barr* in chromosome condensation has been inferred solely from the observation that Barren and topoisomerase II coimmunoprecipitate and interact *in vitro* and *in vivo* (Bhat *et al.* 1996). In addition, mutants in *brn1* and *cnd2* genes, which encode the Barren homolog in budding and fission yeast, respectively, exhibit chromosome

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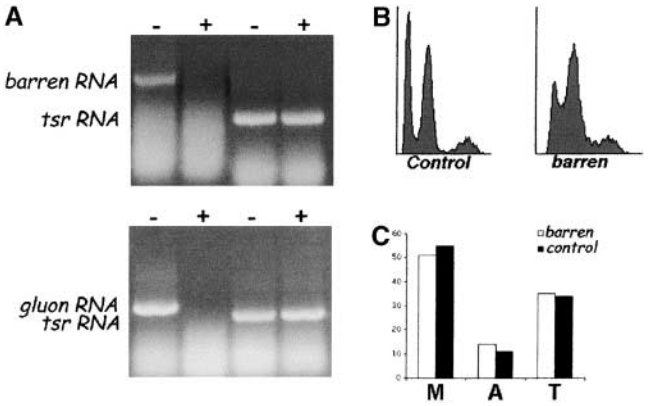

FIGURE 1.—Mitotic parameters in *barr* (RNAi) cells. (A) RT-PCR showing depletion of *barren* and *gluon* transcripts after 72 hr of treatment. +, treated; −, untreated cells. Total RNA was isolated from 1 ml of 72-hr cultures using the RNeasy kit (QIAGEN, Chatsworth, CA). Total RNA was converted into first-strand cDNA using the ImPromII reverse transcriptase kit (Promega, Madison, WI) and the gene-specific antisense primers indicated below. Standard PCR conditions were used, and the *twinstar* (*tsr*) gene was used as an internal control. The GenBank accession number and the sense and antisense gene-specific sequences were as follows: *barren*, no. U74488, sense CAAATAAATGCTGCCGAGGATG, antisense CGAACT TGGTGGGCAATATCAG; *gluon*, no. AF186472, sense AGTT GGACAACA position 2020, antisense TAACACGAACAA position 2754; *twinstar*, no. U08217, sense TTGTTCGTGAAA, antisense ATACGTGTTTCC. The PCR products were analyzed by 1% agarose gel electrophoresis. (B) FACS profiles of control cells and cells treated with *barr* dsRNA. abscissa, DNA content; ordinate, cell number. Note that the profile of *barr* (RNAi) cells is completely different from that of control cells showing a decrease of the 2C peak and an increase of the area between the 2C and 4C peak due to a considerable amount of cells with an aberrant DNA content. FACS analysis was performed as described previously (SOMMA *et al.* 2002). (C) Quantification of mitotic figures. A total of 572 wild-type and 528 *barr* cells were counted. M, percentage of metaphases; A, percentage of anaphases; T, percentage of telophases. Cell cultures, dsRNA production, and RNAi treatments were performed as described previously (SOMMA *et al.* 2002). The GenBank accession number, the sense and antisense gene-specific sequences, and the position of their 5′ nucleotide were the same as for the RT-PCR.

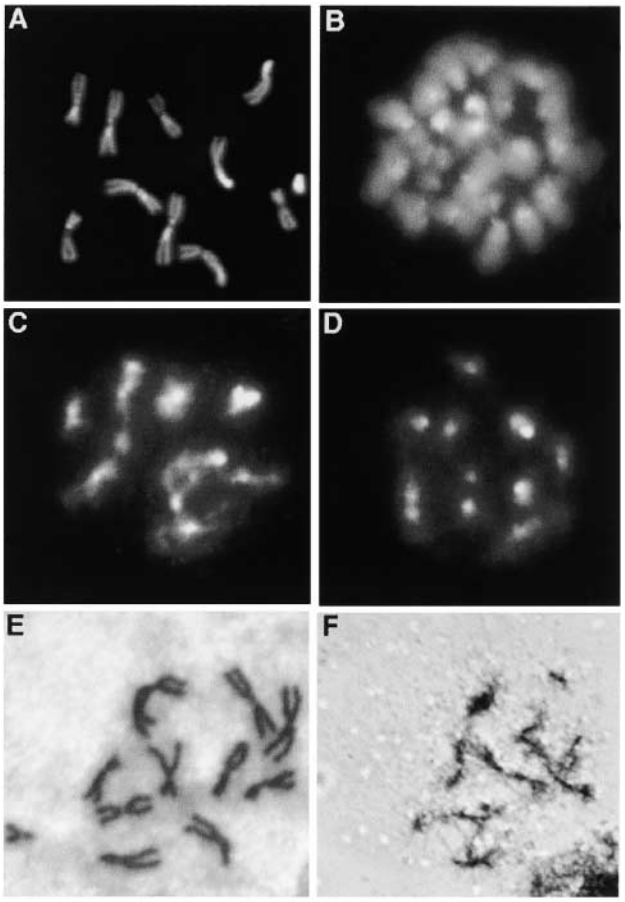

FIGURE 2.—*barren* RNAi interferes with chromosome condensation. (A–D) 4′,6-Diamidino-2-phenylindole (DAPI)-stained S2 metaphases. (E and F) Giemsa-stained S2 metaphases. (A and E) Nontreated control metaphases. (B) *gluon* dsRNA metaphase. (C, D, and F) *barr* dsRNA metaphases. To obtain chromosome spreads, 1 ml of 72-hr cultures was centrifuged at 800 × *g* for 5 min. Pelleted cells were washed in 10 ml PBS and resuspended in 3 ml hypotonic solution (0.5 M Na citrate) for 5 min. Pelleted cells were then fixed in methanol:acetic acid (3:1). A total of 10 μl of this preparation was dropped onto a microscope slide and air dried. Slides were then mounted in Vectashield with DAPI (Vector, Burlingame, CA) to stain DNA and reduce fluorescence fading and/or Giemsa (MERK).

condensation defects (AONO *et al.* 2002; LAVOIE *et al.* 2002). This has led us to ask why Drosophila *barr* mutants do not exhibit the same class of condensation defects revealed by their yeast counterparts.

We have used double-stranded RNA-mediated interference (RNAi) to deplete *barr* function in Drosophila S2 cultured cells. The addition of 15 μg of *barr* double-stranded RNA (dsRNA) into S2 culture cells completely ablated the endogenous mRNA after 72 hr as shown by RT-PCR (Figure 1A). Cytological analysis of treated metaphases revealed that chromosome condensation was highly defective upon depletion of the *barr* gene product (Figure 2). In 98% of metaphases scored (n = 75), condensing chromatin appeared fuzzy and loose. Sister-chromatid morphology was largely disorganized and chromosomes were not distinguishable from one another. This chromatin undercondensation pattern was also detectable during prophase and by using different DNA dyes (*i.e.*, Giemsa, Figure 2F). To assess whether heterochromatin was also influenced by this global decondensation effect, we performed fluorescence *in situ* hybridization (FISH) on metaphase spreads using the dodecasatellite probe. The dodecasatellite probe specifically hybridizes to pericentromeric heterochromatin of chromosome 2 (CARMENA *et al.* 1993). In all *barr* metaphases analyzed (n = 20) the fluorescent signals appeared larger and more diffuse with respect to untreated control metaphases (Figure 3). We measured the area of each fluorescent dot and found that it was,

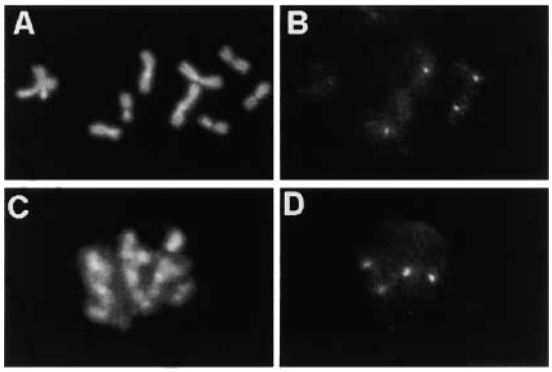

FIGURE 3.—*barr* chromosome decondensation affects pericentromeric regions. Fluorescent *in situ* hybridization with the dodecasatellite probe on untreated (A) and treated (C) metaphases. (A and C) DAPI-stained metaphases. (B and D) FISH. Chromosome spreads for FISH analysis were prepared as described in the legend to Figure 2. The FISH procedure used is described in GATTI *et al.* (1994). Slides were hybridized with a biotin-labeled dodecasatellite probe (CARMENA *et al.* 1993). They were then mounted in Vectashield with DAPI (Vector) to stain DNA and reduce fluorescence fading. Chromosome preparations were analyzed using a Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics, Tucson, AZ). Quantification analysis was performed with the NIH image tool version 1.6 for MacIntosh (http://rsb.info.nih.gov/nih-image).

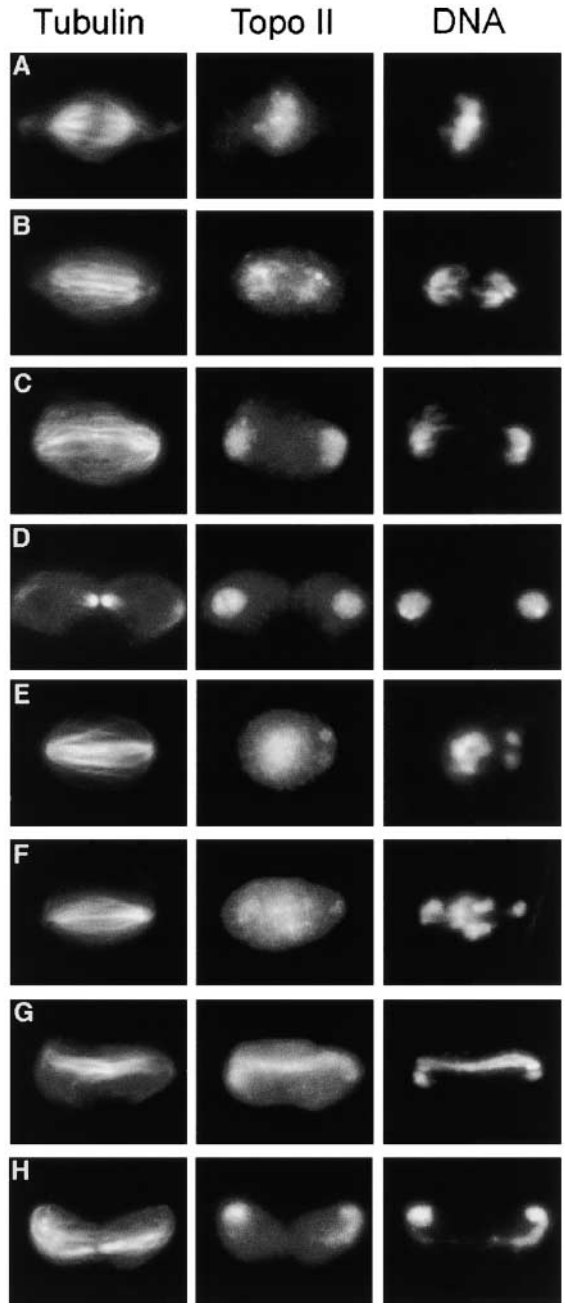

FIGURE 4.—Effects of *barr* dsRNA on S2 mitosis. Cells were stained for tubulin, DNA, and topoisomerase II. (A) Wild-type metaphase. (B and C) Wild-type anaphase. (D) Wild-type telophase. (E) *barr* metaphase. (F and G) *barr* anaphase. (H) *barr* telophase. Note that the topoisomerase II localization is not affected by *barr* dsRNA. Preparation of slides, immunolocalization protocol, and sample analysis were carried out as described previously (SOMMA *et al.* 2002). The antibody dilutions were 1:50 and 1:400 for anti-α-tubulin and antitopoisomerase II antibodies, respectively.

on average, 1.8 times larger in *barr* cells than in controls. This suggests that some degree of chromatin decondensation may also occur at pericentromeric regions. This observation is consistent with Barren localization in the centromeric region (STEFFENSEN *et al.* 2001) where it is thought to be required for the proper function of centromeres. In addition, studies in yeast have shown that *brn-1* may be necessary for the formation of functional mitotic kinetochores (OUSPENSKI *et al.* 2000).

To determine whether the phenotype associated with RNAi of *barr* reflected a general chromatin undercondensation phenomenon or whether it could be attributed directly to depletion of *barr*, we treated S2 cells with *gluon* dsRNA. *gluon* encodes the Drosophila SMC4 homolog and has been shown to be required for chromosome condensation and sister-chromatid resolution (STEFFENSEN *et al.* 2001). We found that the endogenous *gluon* mRNA was completely depleted after 72 hr treatment (Figure 1A) and that its depletion resulted in disruption of chromatin condensation (Figure 1B). Interestingly, the chromatin decondensation pattern in *gluon* dsRNA-treated cells is distinct from that observed in *barr* (Figure 2, B–D), but very similar to that described for *gluon* mutant neuroblast chromosomes (STEFFENSEN *et al.* 2001). Chromosomes in *glu* (RNAi) cells appear swollen and the chromatin downy. In contrast, *barr* chromatin seems unrolled and diffuse and appears to branch off the main chromosome axis, clearly visible in the Giemsa-stained spreads. Moreover, using version 1.6 of the National Institutes of Health (NIH) image tool for MacIntosh (http://rsb.info.nih.gov/nih-image), we measured both chromosome length and width in untreated and treated cells to ask whether axial condensation or completion of the chromatin loops, or both, was af-

fected. For this purpose we focused our analysis on metacentric chromosomes, where both longitudinal and transversal axes were identifiable despite the irregular condensation phenotype. We observed that in *barr* cells the average chromosome length and width did not significantly differ from that of the control (35.40 *vs.* 37.61 pixels and 14.98 *vs.* 12.56 pixels, respectively), whereas *gluon* chromosomes were longer (51.89 pixels) and wider (18.75 pixels) than the control. Collectively, these data suggest that inactivation of these two different subunits (one SMC and one non-SMC) of the condensin complex has diverse effects on chromosome condensation and chromatin loop organization. Furthermore, these observations support the view that components of the condensin complex have distinct, specialized functions (Aono *et al.* 2002).

Chromatin from cells treated with *barr* dsRNA appeared decondensed throughout anaphase and telophase. An extremely high proportion of these figures exhibited chromatin bridges and laggards (98%, $n = 55$, Figure 4, F–H), very likely a consequence of disorganized chromatin fibers. We speculate that the extensive chromosome decondensation may give rise to chromosomes disentangling and failure of chromatid resolution.

We next asked whether these chromatid segregation abnormalities were accompanied by spindle defects. We immunostained treated cells with the anti-α-tubulin antibody and observed that spindle components were not affected by depletion of *barr* and appeared normal during all stages of mitosis (Figure 4). Moreover, mitotic progression of treated S2 cells was not influenced, as the percentage of cells at different stages did not differ significantly from the control (Figure 1C). Furthermore, cytokinesis progressed regularly, as no binucleated cells were observed, indicating that, despite massive anaphase bridging, cell membranes were successfully pinched toward the chromatin bridge. This was also confirmed by fluorescence-activated cell sorter (FACS) profiling of treated cells; no increase of the 8C peak at the expense of the 2C and 4C peaks was observed, as normally occurs when a high proportion of cells become polyploid (Somma *et al.* 2002). The FACS analysis, however, revealed a general reduction of the 2C peak and an increase of the area between the 2C and 4C peaks (Figure 1B). We believe that the expanded area between 2C and 4C peaks represents 2C cells that, despite chromosome condensation defects, complete mitosis and enter the subsequent cell cycle with an aberrant DNA content.

To further characterize the phenotype of *barr* mutant cells, we analyzed whether the localization of DNA topoisomerase II, which has previously been shown to interact with Barr (Bhat *et al.* 1996), was affected in Barr-depleted cells. By immunostaining both mutant and control cells with an antitopoisomerase II antibody, we observed the localization of this enzyme throughout the different stages of mitosis. We found that topoisomerase II was associated with chromatin during prophase, metaphase, anaphase, and telophase in both *barr* and control cells (Figure 4), suggesting that its localization is independent of *barr*. Moreover, this analysis indicates that chromosome decondensation is a direct effect of Barr depletion and not a consequence of DNA topoisomerase II misbehavior.

To date, we do not understand why defects in chromosome condensation are present in Barr-depleted S2 cells and not in mutant embryos. Chromosome compaction was found to be normal in a null mutation of *barren*, so this discrepancy cannot be explained by allele-specific defects. It is possible that *barr* mutant embryos still retain maternal Barr product, which enables chromosomes to condense properly. Alternatively, it is conceivable that the organization of chromatin in embryonic nuclei differs from that of S2 cells, as the former support frequent and rapid mitotic divisions. In this highly dynamic scenario, Barren may not be strictly required for chromatin compaction, or its function may be redundant. One can also argue that the chromatin disorganization observed in S2 cells is a peculiarity of this cell type. We believe this is unlikely, however, as we show that RNAi of *gluon* causes failure in chromosome condensation, a phenotype similar to that elicited by *gluon* mutant neuroblast cells, thus indicating that S2-treated cells do mimic Drosophila mutations. In addition, the observation that dsRNAs of different condensin subunits have different effects on chromosome condensation makes RNA interference a useful tool to molecularly dissect this important aspect of chromosome dynamics.

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