

Mutations in the *Drosophila* Condensin Subunit dCAP-G: Defining the Role of Condensin for Chromosome Condensation in Mitosis and Gene Expression in Interphase

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ABSTRACT

Chromosomes are dynamic structures that are reorganized during the cell cycle to optimize them for distinct functions. SMC and non-SMC condensin proteins associate into complexes that have been implicated in the process of chromosome condensation. The roles of the individual non-SMC subunits of the complex are poorly understood, and mutations in the CAP-G subunit have not been described in metazoans. Here we elucidate a role for dCAP-G in chromosome condensation and cohesion in *Drosophila*. We illustrate the requirement of dCAP-G for condensation during prophase and prometaphase; however, we find that alternate mechanisms ensure that replicated chromosomes are condensed prior to metaphase. In contrast, dCAP-G is essential for chromosome condensation in metaphase of single, unreplicated sister chromatids, suggesting that there is an interplay between replicated chromatids and the condensin complex. In the *dcap-g* mutants, defects in sister-chromatid separation are also observed. Chromatid arms fail to resolve in prophase and are unable to separate at anaphase, whereas sister centromeres show aberrant separation in metaphase and successfully move to spindle poles at anaphase. We also identified a role for dCAP-G during interphase in regulating heterochromatic gene expression.

CHROMOSOMES undergo dynamic behaviors during mitosis to enable the precise separation of the two replicated sister chromatids. It is vital that the replicated sister chromatids are separated successfully. There are two crucial prerequisites for accurate segregation: (1) cohesion between the replicated chromatids must be maintained until anaphase and (2) compaction of the chromosomes into a manageable form, condensation, must be completed prior to metaphase. These processes require two major protein complexes, the cohesin and condensin complexes. Each of these complexes is founded upon a heterodimer of structural maintenance of chromosomes (SMC) proteins, which are chromosome-associated ATPases (HIRANO 1998, 2002). Also within each complex are two or three non-SMC subunits, which attribute specific functions to the SMC holocomplex. Despite a similar structural paradigm, the condensin and cohesin complexes are functionally distinct. Although each complex was originally identified for unique functions during mitosis, it is now clear that both complexes are involved in a wide array of activities, including DNA repair, chromatid separation, and the regulation of gene expression (reviewed in JESSBERGER 2002; HAGSTROM and MEYER 2003; LEGAGNEUX *et al.* 2004).

The structure and function of the cohesin complex is understood in the most detail and its structure has been elucidated (reviewed in HIRANO 2000; LEE and ORR-WEAVER 2001; NASMYTH 2002). The SMC subunits, SMC1 and SMC3, form two antiparallel coiled-coils (HIRANO 2002). One of the two non-SMC subunits, SCC1/Mcd1/Rad21, associates the ends of the SMC coiled-coils into a ring structure (GRUBER *et al.* 2003). This ring structure holds the two sister chromatids together, perhaps by encircling them after S-phase. Cohesin is necessary for holding replicated sister chromatids together from S-phase until anaphase. The complex accumulates on chromosomes prior to S-phase and is maintained and activated through the process of replication. By the end of S-phase, replicated sister chromatids are associated through the cohesin complex at sites along the length of the arms. In yeast, the cohesin complex is maintained until anaphase along the chromosome. In metazoans, the bulk of the cohesin complex is displaced at prophase, but a subset of cohesin complexes is maintained at the centromere and perhaps other sites. This final population of cohesin complexes is lost at anaphase as the sisters separate.

The other SMC complex found in yeast and metazoans that is involved in chromatid segregation is the condensin complex. It also contains two SMC subunits, SMC2 and SMC4 (HIRANO 2002), and three non-SMC subunits, CAP-H, CAP-G, and CAP-D2 (SWEDLOW and

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HIRANO 2003). These three subunits form an 11S regulatory subcomplex that is required to activate the SMC ATPases and to promote mitosis-specific chromatin binding of the holocomplex (KIMURA and HIRANO 2000). However, the individual functions of the non-SMC subunits within the complex remain undefined. Recent studies have identified a second condensin complex containing alternate non-SMC subunits, CAP-G2, CAP-H2, and CAP-D3 (ONO *et al.* 2003). While there is a single condensin complex in both budding and fission yeast, condensin I and condensin II complexes are found in *Xenopus* and humans (ONO *et al.* 2003). Within the *Drosophila* genome, genes coding for a second CAP-H and a second CAP-D2 are found, but there appears to be only a single CAP-G protein (ONO *et al.* 2003).

The condensin I complex was first identified biochemically in *Xenopus* extracts (HIRANO *et al.* 1997). Sperm chromosomes in egg extracts depleted of condensin complex subunits assumed a dispersed interphase organization. When the condensin complex was added back, the chromatin reorganized into condensed chromosomes. This suggested a role in chromosome condensation supported by genetic analyses in yeast. In HeLa cells, depletion of condensin I or II complex subunits disrupt chromosome condensation, but depletion of subunits from both complexes has a more profound effect (ONO *et al.* 2003). Mutations in condensin subunits in yeast show precocious separation of sister chromatids in addition to defects in chromosome condensation (SAKA *et al.* 1994; STRUNNIKOV *et al.* 1995; FREEMAN *et al.* 2000; OUSPENSKI *et al.* 2000; LAVOIE *et al.* 2002). Condensation defects in budding yeast were revealed through the use of fluorescent *in situ* hybridization (FISH) probes to rDNA, which appeared more dispersed in the mutants (STRUNNIKOV *et al.* 1995; FREEMAN *et al.* 2000; LAVOIE *et al.* 2002). In addition, FISH to euchromatic sites in fission yeast revealed loci to be more separated in condensin mutants than in wild type (SAKA *et al.* 1994).

In contrast, genetic analyses in metazoans to date have not delineated an essential role for the condensin complex in chromosome condensation. Embryonic lethal mutations in *barren*, the gene coding for the *Drosophila* homolog of CAP-H, show a failure to separate sister chromatids, but no described defect in condensation (BHAT *et al.* 1996). Animals with larval lethal mutations in *gluon/smc4* also show defects in sister-chromatid separation. A partial effect on condensation is seen by an increase in chromosome width, but no change in the compaction along the length of the chromosomes (STEFFENSON *et al.* 2001). Further complicating the analysis of the role of condensin is the observation that in *Drosophila* S2 cells depleted of Barren by RNAi, chromosomes are poorly condensed with sister chromatids that are fuzzy and indistinct (SOMMA *et al.* 2003). Similarly, depletion of SMC4 by RNAi results in chromosomes that are undercondensed with sister chromatids that are unresolved (COELHO *et al.* 2003). In *Caenorhab-*

ditis elegans, mutations in SMC4 show condensation defects at prometaphase, but little effect on condensation at metaphase and anaphase (HAGSTROM *et al.* 2002). This is similar to observations in chicken cells lacking ScII/SMC2 in which chromosome condensation is delayed, but eventually reaches normal levels (HUDSON *et al.* 2003). Together, these observations suggest that the condensin complex is not the only mechanism for compacting chromosomes in mitosis.

Here we used the genetic analysis of several mutations in the *dcap-g* gene to understand the role of dCAP-G in *Drosophila*. We found that chromosome condensation is compromised during mitosis in *dcap-g* mutant cells, but that normal levels of condensation can be attained by metaphase. This suggests that there is a second pathway for condensing chromosomes that can compensate for a compromised condensin complex. Insight into this pathway comes from our observations that, in the absence of replication, the dCAP-G protein is required for chromosome condensation. In addition, in cells mutant for *dcap-g* sister-chromatid arms are unable to resolve at prophase and sister chromatids show massive bridging defects at anaphase. While there is appropriate assembly of at least two centromere components, aberrant separation at the centromere is observed. Finally, we show that the dCAP-G protein and perhaps the entire condensin complex may be required for chromatin-mediated gene expression in heterochromatic sequences.

MATERIALS AND METHODS

Fly stocks: The *dcap-g*^{K1} and *dcap-g*^{K2} alleles were isolated in a screen that has been previously described (ROYZMAN *et al.* 1997). *EP(2)2346* was obtained from the EP element collection (RORTH 1996) and failed to complement the lethality of *dcap-g*^{K1} and *dcap-g*^{K2}. Two *pimples* alleles, *pim*^{IR46} and *pim*^{IR47}, failed to complement the *pim*¹ allele and one *three rows* allele, *thr*^{IR14}, failed to complement the lethality of *thr*^{1B18}. The *pim*¹, *thr*^{1B1}, and *barren*^{L305} alleles were obtained from the Bloomington Stock Center. The *barren*^{L305} allele was used to make a double-mutant chromosome with *dcap-g*^{K1}. Alleles of *double parked* (*dup*) have been described previously (WHITTAKER *et al.* 2000) and *dup*⁹¹ was used to construct double-mutant chromosomes with *dcap-g*^{K1} and *dcap-g*^{K4}.

Mapping of *dcap-g* and analysis of cDNAs and *dcap-g* gene structure: We screened the *Drosophila* chromosome deficiency kit (Bloomington Stock Center) to identify the region harboring our gene of interest. Both *dcap-g*^{K1} and *dcap-g*^{K2} failed to complement three common deficiencies, *Df(2R)vg33*, *Df(2R)vg56*, and *Df(2R)vg-B*. Preparation of DNA for sequencing was performed as described (WHITTAKER *et al.* 2000) and sequencing was performed at the Massachusetts Institute of Technology Center for Cancer Research, Howard Hughes Medical Institute Biopolymers Laboratory. The *dcap-g* gene is represented by 33 partially and fully sequenced ESTs in the *Drosophila* Gene Collection. Alignment of these ESTs revealed the presence of two alternate splice forms of the *dcap-g* mRNA (Figure 1D).

EP excision and reversion: To show that *EP* element from the line *EP(2)2346* was responsible for the associated lethality, independent transposon excision lines were generated and 17 excision lines reverted the lethality. In addition, 7 excision

lines failed to revert the lethality and produced new *cap-g* alleles, including *dcap-g^{K1}* and *dcap-g^{K5}*, embryonic lethal alleles, and *dcap-g^{K3}*, a semilethal and female-sterile allele. Sequencing of these alleles was performed as above.

Analysis of cytology and immunofluorescence: Embryo preparation and antibody localization were performed as described previously (WHITTAKER *et al.* 2000). Antibodies employed include: primary antibodies to phosphophorylated histone H3 (phospho-Ser10; Upstate Biotechnology, Lake Placid, NY), tubulin (YL1/2, YOL1/34; Sera Lab), MEI-S332 (TANG *et al.* 1998), and CID (BLOWER and KARPEN 2001) and fluorescent secondary antibodies (Jackson ImmunoResearch, West Grove, PA). DNA counterstains used were YOYO-1 and TOTO-3 (Molecular Probes, Eugene, OR). Imaging of embryos was performed using a Zeiss microscope with LSM 510 confocal imaging software (Keck Imaging Facility) and images were processed using Adobe Photoshop.

The cytology of *dcap-g* larval mitotic nuclei was analyzed by DAPI staining of preparations treated in a hypotonic solution of sodium citrate and squashed in acetic acid (GONZALEZ and GLOVER 1993). For immunolabeling, third instar mitotic squashes were prepared and hybridized as described (GONZALEZ and GLOVER 1993). Imaging of larval mitotic squashes was performed using a Zeiss Axiophot microscope and Spot CCD camera and imaging software and images were processed using Adobe Photoshop.

Position-effect variegation: The effects of *dcap-g* alleles on position-effect variegation were determined using the *white* gene allele *w^{mt}*. To look for dominant effects of *dcap-g* alleles, *w^{mt}* males were crossed to *dcap-g* and *barren* lines. White gene expression was scored in female offspring: *w^{mt}/w¹¹¹⁸*; *dcap-g^{K1}/+*, *w^{mt}/w¹¹¹⁸*; *dcap-g^{K2}/+*, *w^{mt}/w¹¹¹⁸*; *barren^{L305}/+*, and *w^{mt}/w¹¹¹⁸*; *CyO/+* siblings. Heads were mounted in Canada Balsam and photographed using a Zeiss Axiophot microscope and Spot CCD camera and imaging software.

The effect of *dcap-g* alleles on the regulation of gene expression by the Polycomb-group response element was studied as described (LUPO *et al.* 2001) using *dcap-g^{K3}* and *dcap-g^{K1}* alleles.

RESULTS

Mutations in *dcap-g* are embryonic lethal and block sister-chromatid separation: The majority of mutations that cause mitotic defects in *Drosophila* do not lead to developmental arrest until the late larval or early pupal stages of development (GATTI and BAKER 1989). This is due to two developmental features of *Drosophila*: (1) during oogenesis large maternal stockpiles of mRNA and proteins needed for cell division are deposited in the egg; and (2) following 16 division cycles in embryogenesis, the majority of cells enter the endo cycle and become polytene. Thus during the larval stages mitosis takes place only in the developing brain and imaginal discs and these tissues are not essential until pupation.

We were interested in identifying mitotic regulatory proteins that are turned over during the cell cycle and must be synthesized *de novo* in each new cycle. We therefore focused on embryonic lethal mutations in which the first 13 nuclear divisions occur normally in a syncytium using maternal supplies, but embryos arrest in the postblastoderm divisions that follow cellularization. During these three divisions, cycles 14–16, zygotic gene

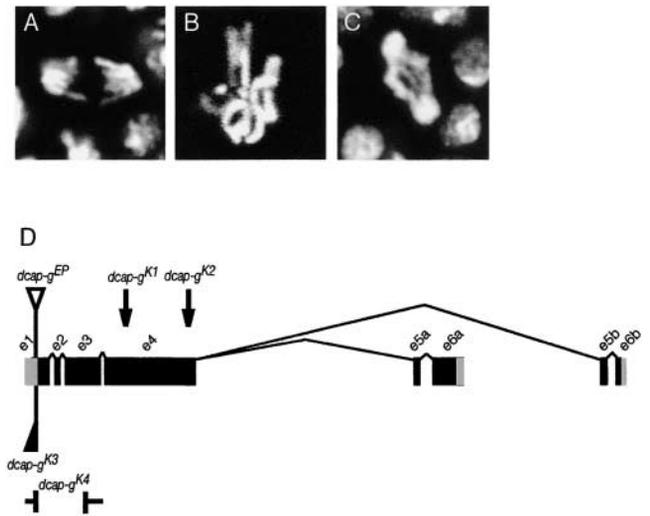


FIGURE 1.—Mutations in genes that code for proteins turned over during the cell cycle are identified in the postblastoderm divisions of *Drosophila* embryogenesis. (A) Wild-type anaphase showing full separation of replicated sister chromatids. (B) An allele of *pimplles* identified in the screen shows a delay in mitosis and a failure to separate sister chromatids. (C) An allele of *dcap-g* (*dcap-g^{K1}*) identified in the screen shows a separation defect. Chromosomes are stained with the DNA dye DAPI. (D) Gene structure of the *dcap-g* gene, indicating two alternate splice forms. Alleles in this article are indicated on the map. Solid boxes represent open reading frames; shaded boxes represent 5' and 3' untranslated regions.

expression occurs and proteins that are degraded at the end of one cell cycle are synthesized *de novo* from the zygotic genome in the next cell cycle. Mitotic arrest during the postblastoderm divisions is a rare phenotype, described for mutations in only four genes prior to this study (LEHNER and O'FARRELL 1989; D'ANDREA *et al.* 1993; EDGAR and DATAR 1996; STRATMANN and LEHNER 1996).

In parallel with an EMS mutagenesis screen for regulators of the G₁-to-S transition (ROYZMAN *et al.* 1997), mutant lines were identified by DAPI staining in which embryos showed an increased number of nuclei with condensed chromosomes that were delayed at the metaphase-to-anaphase transition and failed to separate sister chromatids (Figure 1, A–C). Five mutations on the second chromosome were identified. Genetic complementation analysis for lethality revealed that two of these were alleles of *three rows* and one was an allele of *pimplles*, both known regulators of the metaphase-to-anaphase transition (D'ANDREA *et al.* 1993; STRATMANN and LEHNER 1996). The other two mutations were members of a single, independent complementation group.

Deletion mapping suggested that this complementation group was the *dcap-g* gene, a hypothesis supported by the lethality of these two alleles *in trans* to an *EP* element inserted within the 5' UTR, 17 bp upstream of the start codon of *dcap-g*. We confirmed the identity of these

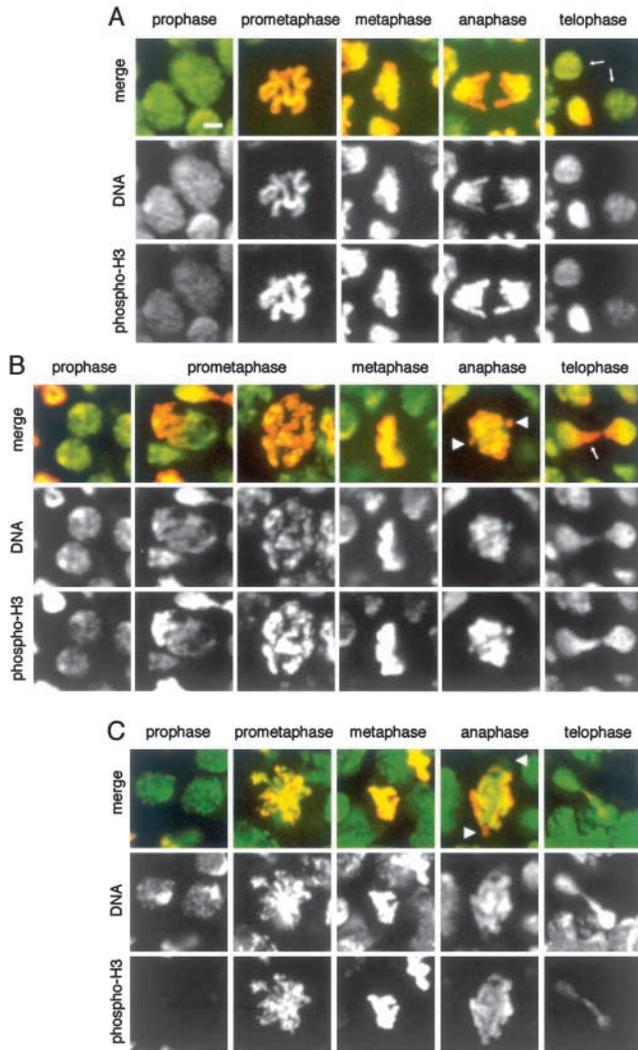


FIGURE 2.—Aberrant chromosome condensation during prophase and the failure to separate sister chromatids at anaphase. Postblastoderm embryos were labeled with an antibody to phospho-H3 and counterstained with the DNA dye, Yoyo. Merged images are shown with DNA in green and phospho-H3 in red. (A) In wild-type cells, prophase cells are beginning to condense (first column). At prometaphase, condensed chromosome arms label strongly with phospho-H3 (second column). Fully condensed chromosomes align at the spindle equator in metaphase (third column). At anaphase, sister chromatids separate fully from their partners and pull to opposite poles of the spindle (fourth column). Upon reaching telophase, chromosomes decondense and gradually lose phospho-H3 staining (fifth column; arrows indicate daughter nuclei). (B) In *dcap-g⁴/Df(2)vg56* mutant embryos, chromatin condenses nonuniformly. Prophase nuclei contain chromatin that appears condensed by DNA staining, but does not label strongly with phospho-H3 (first column). Prometaphase cells were identified using antibodies to tubulin to label the mitotic spindle as it assembles (staining not shown). At prometaphase, cells contain chromatin that is condensed and labeled with phospho-H3, but within the same cell, some chromatin is uncondensed (second column). Prometaphase cells in which most of the chromatin is highly condensed and labeled with phospho-H3 also are seen, but condensation is aberrant (third column). By metaphase, this defect in the level of condensation is resolved and the condensed chromosomes are aligned appropriately on the mitotic spindle (fourth column). A failure

to separate sister chromatids is seen at anaphase and only the fourth chromosome is seen to be separated and have moved to the spindle poles (arrowheads, fifth column) and in telophase, phospho-H3 persists most strongly on the lagging chromatid arms (arrow, sixth column). (C) In *barren^{L305} dcap-g^{K1}* double-mutant embryos, chromatin also condenses nonuniformly in prophase (first column) and prometaphase (second column). By metaphase, chromosome condensation has reached wild-type levels (third column). Chromatid separation defects are seen in anaphase when only the fourth chromosome has clearly separated (one fourth chromosome is visible and indicated by the bottom arrowhead) and lagging chromosomes are seen in telophase with persistent phospho-H3 staining (fifth column). Bar, 5 μ m.

mutations as novel *dcap-g* alleles by two approaches. First, we reverted the lethality of the *EP* element insert by precise excision of the element from the chromosome. Second, we sequenced the *dcap-g* gene in each of the two EMS mutations, *dcap-g^{K1}* and *dcap-g^{K2}*, and identified point mutations that generated premature stop codons in the *dcap-g* coding region (Figure 1D). There is a single *dcap-g* gene in *Drosophila*, but alternative splicing generates two forms of the protein that differ in their C termini. The distal splice form uses exons 5b and 6b (Figure 1D) and codes for a protein that is most homologous to the CAP-G subunits from other organisms. The alternative splice form uses exons 5a and 6a (Figure 1D) and codes for a protein with a C-terminal extension that is not homologous to regions within other CAP-G proteins.

We used the *EP*-element insertion to generate additional *dcap-g* alleles by imprecise excision. We derived seven new lethal alleles and one semilethal, female-sterile allele (Figure 1D). One lethal allele, *dcap-g^{K4}*, is a deletion of 1162 bp at the 5' end of the *dcap-g* gene from the site of the *EP*-element insertion to within the third exon (e3; 1060 bp from the start codon). The female-sterile allele, *dcap-g^{K3}*, is the result of the deletion of most of the *P* element without any loss of *dcap-g* sequence. This allele is not fully viable and shows lethality at the larval stage of development. Viability is reduced to 23% of the expected number of adult males, 64% of adult females.

Mutations in *dcap-g* perturb chromosome condensation during prometaphase and cause a failure to separate sister chromatids at anaphase: The syncytial divisions of the early *Drosophila* embryo are not affected in any of the *dcap-g* embryonic lethal alleles, probably due to the presence of maternal stockpiles from the heterozygous mother. The *dcap-g* alleles do not show any mitotic defects until cycle 15 of the postblastoderm embryo.

In wild-type cells, chromosomes begin the process of chromosome condensation during prophase. At prometaphase, chromosomes are condensed and rod shaped so that individual chromosomes can be visualized with a DNA stain (Figure 2A, second column). Stages were identified in wild-type and mutant cells by the pattern of tubulin

to separate sister chromatids is seen at anaphase and only the fourth chromosome is seen to be separated and have moved to the spindle poles (arrowheads, fifth column) and in telophase, phospho-H3 persists most strongly on the lagging chromatid arms (arrow, sixth column). (C) In *barren^{L305} dcap-g^{K1}* double-mutant embryos, chromatin also condenses nonuniformly in prophase (first column) and prometaphase (second column). By metaphase, chromosome condensation has reached wild-type levels (third column). Chromatid separation defects are seen in anaphase when only the fourth chromosome has clearly separated (one fourth chromosome is visible and indicated by the bottom arrowhead) and lagging chromosomes are seen in telophase with persistent phospho-H3 staining (fifth column). Bar, 5 μ m.

staining (not shown). These chromosomes labeled strongly with an antibody to phosphorylated histone H3 (phospho-H3) that correlates with condensed chromosomes in mitosis (HENDZEL *et al.* 1997). However, in *dcap-g^{K4}* mutant embryonic cells (*dcap-g^{K4}/Df(2R)vg56*), prophase and prometaphase chromosomes showed a range in the level of chromosome condensation. In prophase, some nuclei contained chromatin that appeared condensed with the DNA stain, but labeled only weakly or not at all with the antibodies to phospho-H3 (Figure 2B, first column). As the cells proceeded into prometaphase, the chromosomes became increasingly condensed, but condensation and the accumulation of phospho-H3 was not uniform within the same nucleus. These observations suggest that either the *dcap-g^{K4}* mutants have a prolonged prophase-prometaphase period that allowed us to visualize normal events in the process of chromatin condensation or an unusual level of condensation occurred in the absence of a fully functional condensin complex. However, even the most fully condensed chromosomes observed in prometaphase in the *dcap-g^{K4}* mutants were nonuniformly stained with a DNA dye and unevenly labeled with phospho-H3 (Figure 2, A and B, third column). In embryos carrying mutations in both *dcap-g^{K1}* and *barren^{L305}*, a similar defect in the process of chromosome condensation was observed. In prophase, condensed regions showed little or no phospho-H3 staining (Figure 2C, first column). In prometaphase, chromosomes were abnormally condensed, were not organized into compact rod structures, and showed a nonhomogeneous staining with phospho-H3 (Figure 2C, second column).

To test whether prometaphase was prolonged, we assayed the number of prometaphase figures in *dcap-g^{K4}* mutants compared to those in wild type in representative embryos. We identified wild-type prometaphase figures by the appearance of condensed rod-shaped chromosomes that stained uniformly with phospho-H3. We classified the mutant prometaphase figures by the appearance of any condensed chromosome arms and some degree of labeling with phospho-H3. We scored the appearance of these figures within a single field of cycle-15 mitotic divisions in similar mitotic domains within the dorsal ectoderm of stage-10 embryos. The *dcap-g* mutant embryos have an increased number of prometaphase figures: wild type, 8 ± 1.3 prometaphase figures (within fields from 50 independent embryos); *dcap-g^{K4}*, 23 ± 3.3 prometaphase figures (46 embryos). This suggests that the length of prometaphase may be increased. A similar observation was made for the *barren^{L305} dcap-g^{K1}* double mutant (24 ± 3.5 prometaphase figures; 21 embryos).

At metaphase, condensed chromosomes in wild-type cells align upon the metaphase spindle (Figure 2A, third column). Tubulin staining of the mitotic spindle was used to identify metaphase cells (not shown). We observed apparently normal chromosome condensation

at metaphase in *dcap-g^{K4}* mutant embryos, despite the fact that the pathway leading up to this point was perturbed (Figure 2B, fourth column). Metaphase figures in *dcap-g^{K1}* and *barren^{L305}* double-mutant embryos also showed normal condensation, although the chromosome alignment on the metaphase spindle may be slightly disrupted (Figure 2C, third column).

At anaphase, chromosomes appear normally condensed, but showed defects in sister-chromatid separation. The hypomorphic alleles, *dcap-g^{K1}* and *dcap-g^{K2}*, exhibit bridging of one or two chromosome arms in cycle 15 of embryogenesis (Figure 1C and data not shown). This is similar to the observed phenotype of the embryonic lethal *barren* and *gluon* alleles, although the defect is observed in cycle 16 for mutations in these genes (BHAT *et al.* 1996; STEFFENSON *et al.* 2001; HAGSTROM *et al.* 2002; HUDSON *et al.* 2003). However, the *dcap-g^{K4}* allele exhibited a more severe defect in sister-chromatid separation. In *dcap-g^{K4}* mutant embryos, all of the chromosomes fail to segregate to the spindle poles except for the small 4th chromosome, which can be seen to separate and segregate and appears as a small dot at each of the poles (Figure 2B, fifth column, arrowheads). This severe separation defect is also seen in *barren dcap-g^{K1}* double-mutant embryos (Figure 2C, fourth column). The phenotype of apparently normal condensation at anaphase but a failure of sister-chromatid separation has been observed for mutations in condensin subunits in several metazoans (BHAT *et al.* 1996; STEFFENSON *et al.* 2001; HAGSTROM *et al.* 2002; HUDSON *et al.* 2003). However, the defect seen in the *dcap-g^{K4}* and *barren^{L305} dcap-g^{K1}* double-mutant embryos, in which none of the major chromosomes were able to separate, was much more severe than that reported for other condensin mutants.

As cells enter telophase, the chromosomes gradually decondense and lose phosphorylated histone H3. In *dcap-g* mutants, we observed persistent labeling of the bridging chromosomes with antibodies to phospho-H3 (Figure 2B, sixth column, arrows). A similar observation was made in *barren* mutant embryos (BHAT *et al.* 1996) and in SMC4-depleted S2 cells (COELHO *et al.* 2003). This may represent a defect in the process of decondensation or it may represent a normal stage in the process of chromosome decondensation in which the phosphorylation of histone H3 is lost progressively, beginning at the centromeres and moving along the arms (SU *et al.* 1998). This is consistent with a model in which the arms fail to separate and form the bridges while the centromeres separate appropriately.

These observations reveal a role for the condensin complex in condensation, but show that the cells can compensate to achieve condensation by metaphase. This suggests that surveillance mechanisms may prolong prometaphase until condensation is complete or has reached a sufficient level.

Centromeric proteins localize appropriately during prophase in *dcap-g* mutants, but centromeric attachments are weakened: In chicken cells, mutation of the SMC2 subunit of the condensin complex disrupts the localization of nonhistone chromosomal proteins to the kinetochore (HUDSON *et al.* 2003), in *C. elegans* the localization of kinetochore proteins is aberrant in the absence of a functional condensin complex (HAGSTROM *et al.* 2002; STEAR and ROTH 2002), and in *Xenopus* egg extracts immunodepletion of condensin causes disorganized kinetochore structure and function (WIGNALL *et al.* 2003; ONO *et al.* 2004). In *Drosophila* SMC4-depleted S2 cells, centromeres and kinetochores are able to segregate, while the sister-chromatid arms show bridging at anaphase (COELHO *et al.* 2003). To test whether mutations in *dcap-g* affect the kinetochore and surrounding centromeric chromatin, we examined the localization of two proteins, CID and MEI-S332. MEI-S332 is a centromeric protein that localizes to condensed chromosomes at prometaphase, but concomitant with the separation of sister chromatids, delocalizes from the centromeres at anaphase (MOORE *et al.* 1998). In *dcap-g^{K1}* mutant embryos, MEI-S332 localized normally onto prometaphase chromosomes and properly delocalized at anaphase (Figure 3, A and B). Similarly, MEI-S332 localized to prometaphase and metaphase chromosomes in larval imaginal discs and delocalized at anaphase despite the apparent failure of sister-chromatid separation as evidenced by persistent bridging (Figure 3, C and D). CID, a *Drosophila* CENP-A homolog, localizes to centromeres throughout the cell cycle (BLOWER and KARPEN 2001). We observed that the localization of CID was normal during mitosis in *dcap-g^{K3}* (*dcap-g^{K3}/Df(2R)vg56*) mutant larval imaginal discs (Figure 3, E and F). Centromeres were labeled with CID during prometaphase despite abnormal chromosome condensation. Centromeres were also labeled in metaphase and anaphase. Thus centromere structure is not detectably perturbed by loss of dCAP-G function in *Drosophila*. CID localization also revealed that in mutant anaphases there is normal separation of centromeres despite the failure to separate sister-chromatid arms (Figure 3, E and F).

To elucidate further the role of dCAP-G in chromosome dynamics, we examined the mitotic divisions in the third instar larval brain of animals that are hemizygous for the semilethal *dcap-g^{K3}* allele in squashed preparations and found defects in chromosome morphology. There were several anomalies in mitosis, including aneuploidy, the aberrant separation of centromeres, the failure to resolve sister-chromatid arms, and an increase in the axial length of the chromosomes.

Larval brains from mutant and wild-type animals were treated with a hypotonic solution. In wild type this has the effect of separating the sister-chromatid arms at metaphase while maintaining centromere attachment, thus creating stereotypical mitotic figures (Figure 3G). In the brains of larvae that were hemizygous for *dcap-g^{K3}*, many metaphase

figures had a fewer number of chromosomes than in wild type (Figure 3G), while others were polyploid (data not shown). Strikingly, in these metaphase figures the arms failed to separate and, instead, the DAPI-bright foci at the centromeres appeared to be dissociated (Figure 3G). Anaphase figures that showed chromatid bridging were also observed (Figure 3G). We verified the centromere separation defect using antibodies to the centromeric protein MEI-S332. MEI-S332 localized to the separated centromeres in the mutant metaphase figures (Figure 3I). While this confirmed the identity of the centromere, it also suggested that these nuclei had not yet entered anaphase and that the separation of the centromeres occurred aberrantly in metaphase. This aberrant separation of centromeres was not observed in the *gluon/smc4* larval lethal mutations (STEFFENSON *et al.* 2001) and it was not apparent in the RNAi depletion of Smc4 or Barren (COELHO *et al.* 2003; SOMMA *et al.* 2003).

In addition to abnormal centromere separation, the metaphase figures contained sister-chromatid arms that failed to resolve and therefore the two sister-chromatid arms could not be distinguished (Figure 3, G–I). This suggests that the process of chromatid resolution, a process that normally occurs during prophase as chromosomes condense and the bulk cohesin is released, was disrupted. The failure in sister-chromatid resolution is the likely upstream defect that leads to the segregation errors in anaphase, such as lagging chromosomes, bridging, and, ultimately, aneuploidy.

Mutations in *gluon/smc4* show minor defects in chromosome condensation during the larval mitotic divisions in brains in that the width of the chromosomes is broader (STEFFENSON *et al.* 2001). The *dcap-g^{K4}* mutants showed no measurable change in the width of the mitotic chromosomes, yet measurements of the length of the X chromosome, which was easily identified due to the presence of the DAPI-bright heterochromatin at one end of the chromosome (arrowheads in Figure 3G), revealed a slight increase in axial length from 2.79 μm in wild type (standard deviation of 0.61 μm ; $n = 13$) to 3.82 μm in *dcap-g* mutants (0.83 μm ; $n = 27$). A greater defect in axial condensation was observed with the two autosomes, chromosomes 2 and 3. These were identified by the DAPI-bright heterochromatin in the center of the arms (arrows in Figure 3G). The combined average length of these chromosomes was 4.25 μm ($\pm 0.6 \mu\text{m}$; $n = 25$) in wild type and 7.8 μm ($\pm 1.1 \mu\text{m}$; $n = 18$) in *dcap-g* mutants. However, there is no complete loss of the chromosome condensation at metaphase. This difference in the *gluon* and *dcap-g* phenotypes may reflect the roles of the SMC *vs.* non-SMC subunits in the condensin complex, differences in allele strengths, or differences in protein stability during the cell cycle. An examination of the DAPI-bright heterochromatic DNA on the autosomes revealed that this region was longer in the *dcap-g^{K4}* mutants (Figure 3H). DAPI preferentially

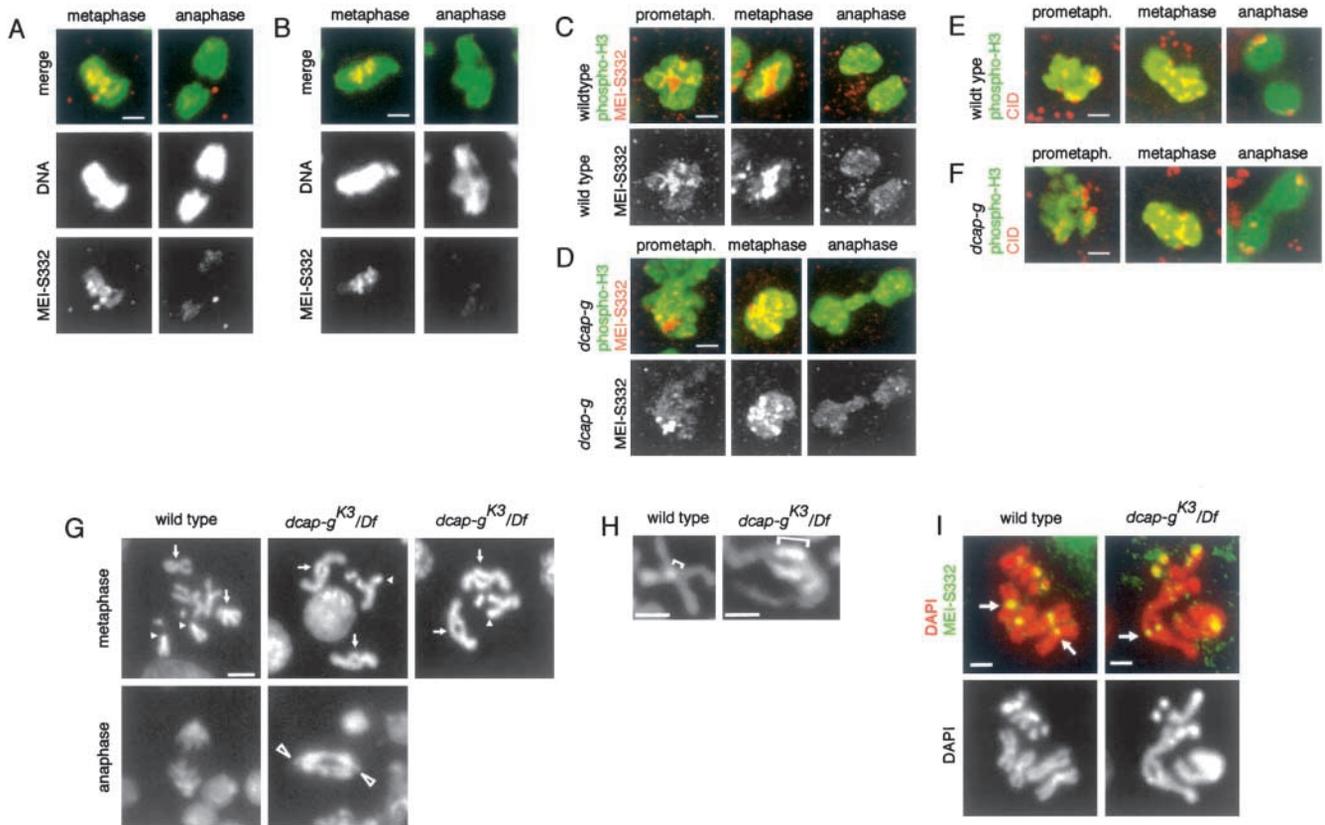


FIGURE 3.—Centromere organization and dynamics are unperturbed in the mutant *dcap-g* cell cycle. (A) In wild-type embryos, MEI-S332 antibody localizes to the centromere at prometaphase and metaphase (first column); however, the protein delocalizes as sister chromatids separate at anaphase (second column). (B) In *dcap-g^{K3}/Df(2)vg56* mutant embryos, MEI-S332 localizes normally at metaphase (first column) and delocalizes from the centromeres at anaphase even as sister-chromatid separation fails and bridging is observed. (C) MEI-S332 protein localizes to centromeres in mitotic nuclei in the larval brain at prometaphase and metaphase and is displaced at anaphase. The MEI-S332 antibody is red (overlay appears yellow) and DNA is green in the merged image. The MEI-S332 antibody alone is shown below. (D) In *dcap-g^{K3}/Df(2)vg56* larvae, MEI-S332 is seen at the centromeres at prometaphase even though the chromosomes are not fully condensed. Localization of the protein persists through metaphase, but the protein is delocalized during anaphase despite the failure in sister-chromatid separation. The MEI-S332 antibody is red (overlay appears yellow) and the DNA is green in the overlay. MEI-S332 alone is shown below. (E) The centromere-specific histone variant, CID, localizes to the centromeres throughout the cell cycle and is seen here in prometaphase, metaphase, and anaphase. (F) In *dcap-g^{K3}/Df(2R)vg56* mutant larvae, CID localization appears normal even in an undercondensed prometaphase figure and persists through metaphase. At anaphase, centromeres labeled with CID have successfully separated in *dcap-g* mutant cells. Bar, A–F, 5 μm . (G) Larval mitotic squashes of *dcap-g^{K3}* mutants. In wild-type larvae, DAPI-stained nuclei in metaphase show replicated sister chromatids that are associated at the centromeres, but not along the arms. In *dcap-g^{K3}/Df(2)vg56* larvae, nuclei in metaphase show sister chromatids that are associated along the length of the arms, but separated at the centromeres. Arrows indicate the centromeres of the autosomes and arrowheads indicate the centromeres of the X chromosomes. Centromeres were identified by brighter DAPI staining. In addition, the chromosomes from *dcap-g^{K3}/Df(2)vg56* larvae are longer than those in wild-type larvae. Wild-type anaphase figures show chromosomes being pulled to spindle poles. In *dcap-g* mutant figures, there is chromosome bridging and only the fourth chromosomes have successfully segregated to opposite poles. Bar, 5 μm . (H) DAPI preferentially stains AT-rich sequences found in centromeric heterochromatin. These centromeres, shown at a higher magnification, reveal that the DAPI-bright region, and thus a portion of the heterochromatin, is longer in *dcap-g^{K3}* mutant larvae. Bar, 2 μm . (I) Centromeric regions were identified by bright DAPI staining (red) and confirmed with antibodies to MEI-S332 (green). In a wild-type metaphase figure, MEI-S332 is localized to the attached sister centromeres. In a *dcap-g^{K3}/Df(2)vg56* mutant metaphase figure, MEI-S332 is localized to separated centromeres. Bar, 2 μm .

stains repetitive, AT-rich sequences of the DNA that are found on all *Drosophila* chromosomes at the centromeres and along the Y chromosome (DONNELLY and KIEFER 1986; KAPUSCINSKI 1995). This suggests that condensation of the heterochromatic DNA at the centromere is disrupted, although this defect alone does not account for the overall increase in chromosome length.

Our *dcap-g* mutations reveal distinct, but perhaps interrelated, roles for dCAP-G: resolution of sister-chromatid arms, association of sister centromeres, and a contributing, but not exclusive role, in axial chromosome condensation.

The condensin complex is essential for condensation in the absence of a replicated sister chromatid: The differ-

essential requirement of the condensin complex in chromosome condensation, as suggested by the condensin mutant phenotypes in *Drosophila* and *C. elegans* and the *in vitro* studies in *Xenopus* extracts, may be due to the different source of the chromosomes in the different systems. While in the *Drosophila* and *C. elegans* studies the endogenous chromosomes were analyzed, the *Xenopus* experiments used exogenous sperm chromatin in egg extracts. Sperm chromatin contains protamines that must be replaced by histones before undergoing condensation. In addition, in the *Xenopus* studies condensation is measured in the absence of replication, and thus single sister chromatids are condensed (HIRANO *et al.* 1997). Topoisomerase II (TopoII) is a component of the chromosome scaffold (EARNSHAW *et al.* 1985; GASSER *et al.* 1986) and TopoII mutants show a similar phenotype to condensing mutants (DINARDO *et al.* 1984; HOLM *et al.* 1985; UEMURA *et al.* 1987). Recent studies in *Xenopus* have shown a role for DNA replication in the recruitment of topoisomerase II to the chromosomes to facilitate condensin assembly and condensation (CUVIER and HIRANO 2003). Furthermore, in S2 cells depleted of SMC4, topoisomerase II is not localized normally and Barren is not loaded onto chromosomes (COELHO *et al.* 2003).

We wanted to test whether the presence of a replicated sister chromatid could augment condensation, explaining why the condensin complex was not essential for condensation in the *Drosophila* mutants. We analyzed the requirement for *dcap-g* for condensation in the absence of a sister chromatid by employing a mutant in an essential replication initiation factor, *double parked* (*dup/cdt1*). Mutant alleles of *dup* block replication in cycle 16 of the postblastoderm divisions (WHITTAKER *et al.* 2000; GARNER *et al.* 2001). *dup^{at}* mutants fail to replicate in S-phase, yet proceed into mitosis and often appear clustered at the spindle equator in a pseudometaphase due to the attachment of the single kinetochore to microtubules emanating from both spindle poles (Figure 4; PARRY *et al.* 2003). Cells accumulate in mitosis, but fail to complete anaphase, as the single kinetochores are incapable of a normal bipolar attachment and induce the spindle checkpoint (PARRY *et al.* 2003). The chromosomes, although composed of single sister chromatids at cycle 16, condense appropriately and show robust labeling with phospho-H3 (WHITTAKER *et al.* 2000; GARNER *et al.* 2001). We found that condensation in the *dup* mutant was dependent on a functional dCAP-G protein. In *dcap-g^{K1} dup^{at}* double mutants, cells at cycle 16 contain unreplicated chromosomes that failed to condense into discernible metaphase chromosomes and showed punctate labeling with phospho-H3 (Figure 4). This is in striking contrast to chromosomes composed of two replicated sister chromatids and shows that, in the absence of replicated sister chromatids or the process of DNA replication, the condensin complex is essential for chromosome condensation.

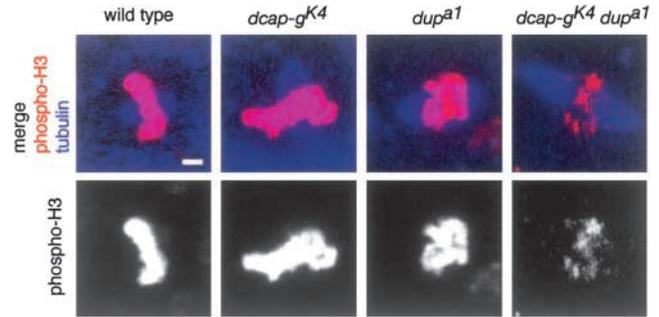


FIGURE 4.—A failure to replicate chromosomes reveals a condensation defect in *dcap-g* mutants. Postblastoderm embryos were labeled with an antibody to phospho-H3 (red) and antibodies to tubulin (blue). Below the merged image appears the phospho-H3 staining. Embryos were examined at stage 11 and cells in mitotic division 16 were imaged. In wild-type embryos, metaphase chromosomes in cycle 16 are fully condensed. In *dcap-g^{K4}/Df(2)vg56* embryos, chromosomes in metaphase of cycle 16 are also condensed. In *dup^{at}* homozygous embryos, a failure to replicate chromosomes in the prior S-phase results in mitotic figures in mitosis 16 that contain single chromatids that are fully condensed. Embryos that are homozygous mutant for both *dcap-g^{K4}* and *dup^{at}* reveal a condensation defect in mitosis 16. Chromosomes are diffuse and phospho-H3 staining is significantly diminished and nonuniform. This demonstrates that the mitotic chromosomes in *dcap-g* mutant embryos have not condensed properly in the absence of prior DNA replication.

dCAP-G has a role in interphase in controlling gene expression: Adult flies carrying the *dcap-g^{K3}* (*dcap-g^{K3}/Df(2R)vg56*) allele exhibited phenotypes suggestive of defects in gene expression such as wing notches and rough eyes (data not shown). While staining with acridine orange revealed comparable levels of cell death in the imaginal discs of male and female larvae, the wing notches appeared only in the adult male flies (data not shown). This suggested that the defects in this tissue may be the result of disrupting male-specific gene regulation. We tested the role of dCAP-G in regulating gene expression by examining the effect of *dcap-g* alleles on position-effect variegation (PEV). PEV is the effect on gene expression mediated by the chromatin structure associated with heterochromatic regions. The *white^{mth}* allele is the result of a genomic inversion that places the *white* gene next to heterochromatic DNA and results in a downregulation of gene expression to produce white patches in the eye (Figure 5A). We found that embryonic lethal alleles of *barren* and *dcap-g* exhibited a dominant suppression of PEV at the *white^{mth}* locus. Thus, one copy of *dcap-g^{K2}* (Figure 5B) or *dcap-g^{K1}* (Figure 5C) or *barren^{L305}* (Figure 5D) resulted in an increase in red pigment due to an increase in *white* gene expression.

Barren was previously shown to interact with the Polycomb complex, a protein complex that maintains a repressive chromatin structure (LUPO *et al.* 2001). This complex acts at specific recognition elements, one of which is the *FAB-7 Polycomb response element (PRE)*. *FAB-7*

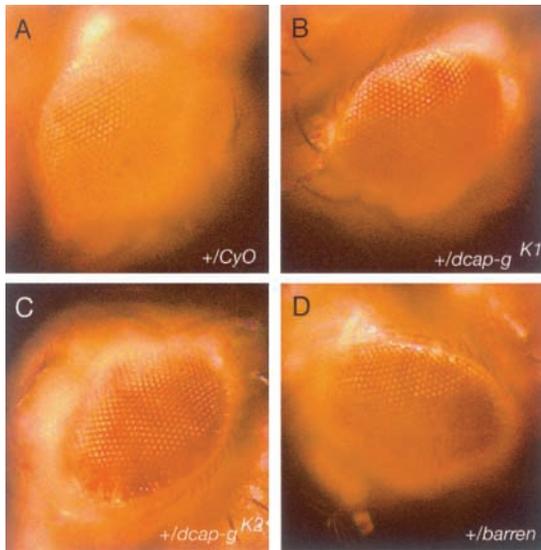


FIGURE 5.—Alleles of *dcap-g* exhibit a dominant suppression of position-effect variegation. Five-day-old females are shown. (A) The *white* gene in the *w^{mh}* background is repressed as a result of its proximity to heterochromatic regions on the *X* chromosome and the eye appears white. These flies are from the same cross as in B, but carry the balancer chromosome instead of a *dcap-g* allele. (B) Heterozygous *dcap-g^{K1}* females show a dominant derepression of the *white* gene and the eye is red. (C) Heterozygous *dcap-g^{K2}* females show a dominant derepression of the *white* gene. (D) One copy of *barren^{L305}* also shows dominant derepression of the *white* gene.

PRE represses expression of an adjacent *white* gene in a transgene construct, but this repression is alleviated by mutations in members of the Polycomb complex or by mutations in *barren*. As an additional test of the role of dCAP-G in interphase gene expression, we asked whether *dcap-g* mutations affected repression by *FAB-7 PRE*, but we did not see any such effect.

DISCUSSION

In these studies we have demonstrated a role for dCAP-G in chromosome condensation and cohesion in *Drosophila*. The dCAP-G protein is the *Drosophila* homolog of the CAP-G subunit of the condensin complex whose activity has not been studied in metazoans prior to this work. We illustrate a requirement for dCAP-G in the process of condensation during prophase and prometaphase; however, compensatory mechanisms ensure that chromosomes condense prior to metaphase. We also show that the reorganization of chromatin into condensed chromosomes is a process that involves the prior replication of chromatids and the condensin complex. Anaphase defects are also observed; specifically, sister-chromatid arms fail to separate. The separation defect is likely the result of the defect in sister-chromatid resolution during prophase that was evident in neuroblast mitotic squashes. In contrast, centromere separation is observed at anaphase and, in larval neuroblast prepara-

tions, this separation occurs aberrantly in metaphase figures. The role of dCAP-G, and possibly of the condensin complex, is not limited to mitosis. We have identified mutations that reveal roles for dCAP-G during interphase in heterochromatic gene expression.

Mutations in *dcap-g* suggest that a checkpoint monitors condensation in prometaphase: There is a striking condensation phenotype early in the cell cycle in *dcap-g* mutants. In prophase and prometaphase, condensation is non-uniform. By metaphase, condensation has achieved apparently normal levels, suggesting that a prolonged prometaphase enables chromosomes to achieve a high degree of condensation in the absence of a fully functional condensin complex. The role of the condensin complex in chromosome condensation prior to metaphase has been observed in other organisms. Mutations in the *C. elegans smc-4* gene diminish chromosome compaction at prometaphase, but chromosomes are highly condensed by metaphase (HAGSTROM *et al.* 2002). RNAi depletion of SMC4 in *Drosophila* S2 cells also shows aberrant condensation at prometaphase (COELHO *et al.* 2003). Similarly, in chicken cells lacking the condensin subunit ScII/SMC2, chromosome condensation is delayed, but chromosomes ultimately reach nearly normal levels of condensation (HUDSON *et al.* 2003). Recently it has been shown that *Xenopus* and humans have two sets of condensin subunits (ONO *et al.* 2003). It is possible that in some organisms when one complex is unable to function the other can compensate partially and complete chromosome condensation by metaphase. However, while there are two CAP-H and CAP-D2 condensin subunits in *Drosophila*, there is only a single gene coding for a CAP-G subunit (ONO *et al.* 2003). This single dCAP-G protein may be required in both complexes; therefore we would not expect such compensation to occur in *Drosophila*. The significance of the alternate splice forms of the *dcap-g* gene is not known, although the two predicted proteins are similar across most of their lengths.

The prolonged prometaphase may be the result of activating the spindle checkpoint. This checkpoint may be used to monitor the degree of condensation at prometaphase to prevent sister-chromatid separation prior to complete condensation. The spindle checkpoint monitors the kinetochore-spindle attachment and delays anaphase until the appropriate bipolar connections are achieved and the chromosomes are congressed at the metaphase plate (reviewed in CLEVELAND *et al.* 2003). It is thought that this checkpoint might monitor the tension at the kinetochores. It is possible that chromosome condensation might be monitored through this pathway, as a hypocondensed centromere and/or chromosome might reduce tension. In this way, the spindle checkpoint may delay progression through the cell cycle until the chromosomes are sufficiently condensed. The spindle checkpoint would require two sister chromatids for bipolar attachment, tension, and congression to occur.

The observation of a severe condensation defect at metaphase in other systems may be due to the absence of an active spindle checkpoint. For example, RNAi of Barren, the CAP-H homolog in *Drosophila*, demonstrated chromosome condensation defects at metaphase (SOMMA *et al.* 2003), but S2 cells have weak checkpoints controlling behavior in mitosis (ADAMS *et al.* 2001).

Coordination between replication and condensation: The *dcap-g dup* double mutants show that the condensin complex is dispensable for chromosome condensation by metaphase except in the absence of a replicated sister chromatid. What could replication provide to the condensation process? The cohesin complex could compensate for a faulty condensin complex, and replication is required to assemble the cohesin complex and establish cohesion. In this model the unreplicated sister chromatids in *dup* mutants would contain inactive cohesin complex that would be unable to compensate for a faulty condensin complex in the process of chromosome condensation. Alternatively, experiments in *Xenopus* show that TopoII activity during replication is a prerequisite for setting up a structural axis required for the mitotic chromosome assembly (CUVIER and HIRANO 2003; SWEDLOW and HIRANO 2003).

Although there are no mutations in TopoII in *Drosophila*, some insights into whether TopoII could play the same role during replication in establishing a condensation-competent chromosome axis emerge from RNAi ablation in *Drosophila* cell culture. In S2 cells depleted of TopoII, mitotic chromosomes condense, but chromosomes are less compact at the metaphase plate (CHANG *et al.* 2003). In these TopoII-depleted cells, Barren loading to centromeres and its dissociation at anaphase are normal and chromosome decondensation begins at anaphase (CHANG *et al.* 2003). In SMC4-depleted S2 cells, TopoII localization is aberrant. In cells containing SMC4, TopoII appears in discrete regions along a defined chromatid axis, while in SMC4-depleted cells TopoII is associated diffusely with the chromosomes (COELHO *et al.* 2003). Thus, perhaps we see a condensation defect at metaphase in the *dup dcap-g* double mutants because there was no replication, TopoII was unable to establish the appropriate architecture prior to mitosis, and the condensin complex is faulty during mitosis.

Roles of condensin at centromeres in controlling segregation and interphase gene expression: Our studies present evidence that there is a distinct role for dCAP-G in centromere segregation. The *dcap-g, barren, gluon*, as well as yeast and *C. elegans* condensin mutants exhibit the segregation of centromeres at anaphase, but a failure to separate arms. We find aberrant separation of centromeres prior to anaphase, at metaphase, in *dcap-g* mutants. Few other mutants show this defect in sister-chromatid centromere association. Mutations in cohesin subunits show premature separation of both the arms and the centromeres of chromatids (reviewed in LEE and ORR-WEAVER 2001). In *Drosophila*, mutations in

wings apart-like (wapl) cause aberrant separation of centromeres. However, in *wapl* mutants, the arms are resolved appropriately (VERNI *et al.* 2000). This cytological observation suggests that there is a role for dCAP-G and perhaps the condensin complex in mediating centromere association via heterochromatin. An effect on heterochromatic chromosome condensation in *dcap-g* mutants is further suggested by our observation that DAPI-bright repetitive sequences at the centromere appeared to be expanded. A distinct role for dCAP-G at the centromeres of the chromosomes is consistent with observations in other organisms that the condensin complex accumulates at centromeres (SUTANI *et al.* 1999; STEFFENSON *et al.* 2001; HAGSTROM *et al.* 2002).

Consistent with the concept of a specific role for condensin in centromeric genomic regions is the observed role of condensin in regional gene regulation. We found that dCAP-G is required for the transcriptionally repressive state of centromere-proximal heterochromatin. Mutations in *wapl* not only show premature separation of centromeres at metaphase, but also, like *dcap-g*, act as dominant suppressors of variegation at the *white* locus (VERNI *et al.* 2000). This emphasizes the intrinsic relationship between mitotic centromere structure and interphase heterochromatic organization. This is consistent with the widely held notion that the transcriptionally inactive state of mitotic condensation may be similar to the transcriptionally repressed heterochromatic regions of the genome. It is now becoming clear that the same proteins may establish both chromatic states.

Global gene repression in *C. elegans* is observed in XX hermaphrodites that downregulated gene expression from both X chromosomes. Dosage-compensation factors that resemble condensin subunits form a complex that associates with the chromosomes and mediate this chromosome-wide gene regulation. In *C. elegans*, a condensin complex containing MIX-1, SMC-4, and HCP-6 mediates mitotic chromosome condensation and a condensin-like complex containing MIX-1, DPY-26, DPY-27, and DPY-28 is required for dosage compensation (reviewed in HAGSTROM and MEYER 2003). Silencing at the mating-type loci in *Saccharomyces cerevisiae* has also been found to require condensin subunits, specifically, CAP-D2 and SMC4, but not SMC2 (BHALLA *et al.* 2002). Perhaps in yeast, where there is a single condensin complex, a subset of condensin proteins assembles into a distinct condensin-like complex that is required for transcriptional silencing.

Conclusions: These studies reveal several distinct roles for the dCAP-G condensin protein. In addition to its role in condensation and sister-arm resolution, our observations highlight the role of dCAP-G and perhaps the condensin complex in centromere organization. This role is important for the association of sister chromatids during mitosis and for the regulation of heterochromatin-mediated gene expression during interphase.

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