The EMBO Journal Vol.17 No.4 pp.1063–1075, 1998

Mutations in *ccf*, a novel *Drosophila* gene encoding a chromosomal factor, affect progression through mitosis and interact with Pc-G mutations

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We report herein the isolation of *ccf*, a new gene located in region 82E and essential for *Drosophila* development. This gene, expressed throughout development, encodes a novel product of 68 kDa which is found in the nucleus during interphase and labels, in a novel pattern, centrosomes and chromosome arms during mitosis. Mutations in *ccf* give rise to late larvae with small imaginal discs and to adults showing appendages of reduced size, consistent with CCF involvement in cell proliferation. Neuroblast squash analyses show that CCF is required for proper condensation of mitotic chromosomes and, therefore, for progression through mitosis. Furthermore, we observe that adult *ccf* mutants as well as animals overexpressing CCF during larval stages exhibit homeotic transformations. We also find that mutations in the Pc-G genes *Polycomb*, *polyhomeotic* and *Enhancer of zeste* are enhanced by *ccf* mutations. Finally, we show that the CCF protein binds to specific sites on polytene chromosomes, many of which are shared with the Posterior sex combs Pc-G protein. Together, these results suggest a role for the CCF protein in the maintenance of chromosome structure during mitosis and interphase.

Keywords: centrosome/chromosome condensation/*Drosophila*/homeotic transformation/mitosis

Introduction

In *Drosophila*, essential cell cycle functions have been identified by mutation analyses and classified tentatively into three groups, on the basis of their phenotypic effects (for reviews, see Glover, 1991; Gonzalez *et al.*, 1994; Orr-Weaver, 1994). The first class corresponds to genes required only for early development, whose mutations produce a maternal-effect lethal phenotype (Foe *et al.*, 1993). Some of these maternally expressed genes encode products specifically required for pre-blastoderm and syncitial divisions. The second class of mutations leads to embryonic death and identifies genes required, mainly as regulators, for post-cellularization divisions (Foe *et al.*, 1993). *string*, a *Drosophila* homolog of fission yeast *cdc25* (Edgar and O'Farrell, 1989; Jimenez *et al.*, 1990), *cyclin A* (Lehner and O'Farrell, 1989) and *cyclin B* (Lehner and O'Farrell, 1990; Knoblich and Lehner, 1993) exemplify this class of genes. The third class of cell cycle mutants is defined by late larval or early pupal lethality, combined with defective imaginal development (Gatti and Baker, 1989). This late lethality is frequent among cell cycle mutants, as the maternal contribution of most components required for cell proliferation appears to be sufficient for survival throughout embryonic cycles. As a result, the lack of such components becomes limiting only during post-embryonic development.

Chromosome condensation is the first step in mitosis and is critical for faithful segregation of genetic material. To date, few participants in chromosome condensation have been identified in eukaryotes, and their actual contribution to the process is still highly debated. DNA topoisomerase II, as the major component of the chromosome scaffold (Earnshaw *et al.*, 1985; Gasser *et al.*, 1986), is thought to participate in higher order chromosome structure, acting as a loop fastener by directly interacting with specific DNA sequences called scaffold-associated regions (Gasser and Laemmli, 1987). Hyperphosphorylation of histone H1 has been shown to accompany chromosome condensation in mitosis (for review, see Hohmann, 1983) and was thus proposed to trigger this process directly (for review, see Bradbury, 1992), but this role is still controversial (Roth and Allis, 1992; Ohsumi *et al.*, 1993; Guo *et al.*, 1995). More recently, a novel family of proteins has emerged, referred to as the SMC family (structural maintenance of chromosomes), whose members are motor proteins involved in chromatin compaction (for review, see Hirano *et al.*, 1995). Owing to the successful isolation of mitotic chromosomes, members of this family have been identified readily as essential factors for mitotic chromosome condensation (Hirano and Mitchison, 1994; Saka *et al.*, 1994). Interestingly, SCII, a major scaffold-associated protein, is a member of the SMC family (Saitoh *et al.*, 1994).

Chromatin structure is also important for gene expression, which is particularly well illustrated in *Drosophila*. The respective identities of body segments in the fly are set up by the coordinated action of homeotic genes of the HOM-C complexes. The expression of these homeotic selectors is positively and negatively regulated, both for their initiation by segmentation genes and for their maintenance by genes of the trithorax (trx-G) and Polycomb group (Pc-G) (for review, see Bienz and Müller, 1995). Products of the Pc-G genes are chromosome-associated repressors, while trx-G gene products are activators (reviewed in Kennison, 1995). Pc-G genes help to

maintain the expression of homeotic genes within their appropriate boundaries. Loss-of-function mutations in this class of genes lead to ectopic expression of homeotic genes and therefore to body segment transformations in the embryo, as well as in the adult. It has been shown that several Pc-G proteins bind to common specific sites on polytene chromosomes (DeCamillis *et al.*, 1992; Rastelli *et al.*, 1993; Carrington and Jones, 1996). Moreover, some of them have been found to be associated in a multiprotein complex (Franke *et al.*, 1992). A proposed role for this complex is to drive local chromatin packaging and silencing of the targeted loci by preventing the binding of transcriptional activators (Zink and Paro, 1995).

Here, we describe a novel *Drosophila* gene which is expressed throughout development. The product of this gene is nuclear in interphasic cells and is a centrosomal and chromosomal factor (CCF) during mitosis. Loss-of-function mutations define *ccf* as a larval–pupal lethal cell cycle mutant. We present evidence for its involvement during the process of mitotic chromosome condensation. Our results also suggest that CCF could be involved in regulation of gene expression through the chromatin structure, as its deregulation provokes the appearance of homeotic phenotypes, and *ccf* loss-of-function mutations enhance Pc-G mutations.

Results

Isolation, cloning and temporal expression of the ccf gene

In an effort to isolate novel genes essential for *Drosophila* development, we focused on an enhancer trap line, called A46, whose reporter gene was expressed during embryogenesis. The A46 enhancer trap line was first described as a homozygous viable P(*lacZ*, *ry*) insertion in locus 42A (O'Kane and Gehring, 1987). The actual cytological position is locus 82E on chromosome 3R. Genomic DNA flanking the A46 insertion was recovered, and subsequent cloning steps resulted in the constitution of a 65 kb contig (Figure 1A). A transcription unit located near the A46 element was detected by Northern blot analyses of the cloned region (Figure 1B). This transcription unit, which is the only one detected in this systematic analysis, defines the *ccf* gene.

Genomic and cDNA probes from *ccf* were used for Northern blotting analyses on poly(A)$^+$ mRNA from a series of developmental stages. They revealed two transcripts of 3.2 and 3.6 kb which are expressed throughout development (Figure 1C). Only the shorter transcript is present in adult females and in pre-blastoderm embryos, indicating a marked maternal contribution to early zygotic development. In all subsequent developmental stages, both transcripts are present.

Several clones were recovered from 0–4 h (enriched in maternal cDNAs) and 4–8 h (zygotic cDNAs) NB40 cDNA libraries (Brown and Kafatos, 1988). Extensive restriction analyses showed that the difference between the two classes of cDNA clones fell in the 5′-most region, where the 4–8 h full-length clones are ~400 bp longer than the 0–4 h clones (Figure 1B). This strongly suggests that two different transcription start sites are used to generate the *ccf* mRNAs. In addition, we found that clones from both sources were co-linear with the corresponding genomic DNA, supporting the idea of an intronless structure for the *ccf* gene.

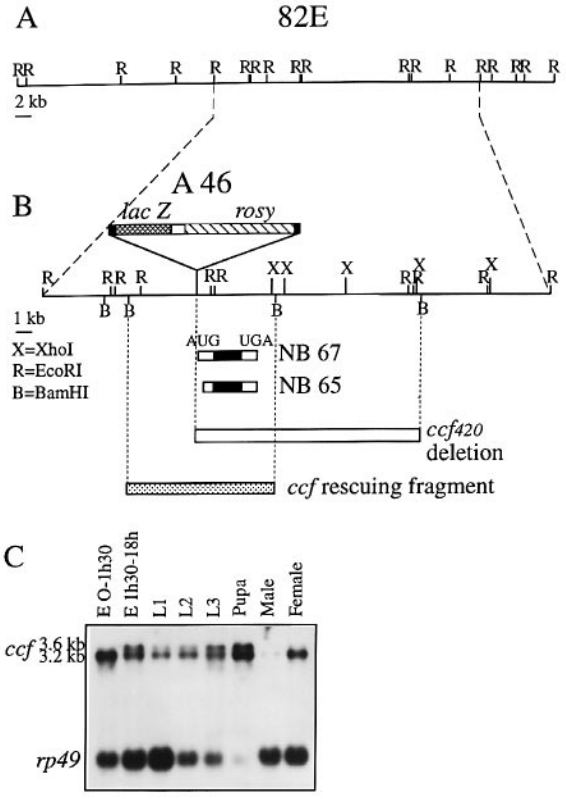

Fig. 1. Molecular cloning and temporal expression of the *ccf* gene. (**A**) *Eco*RI restriction map of the 65 kb contig encompassing the *ccf* unit in region 82E. (**B**) Detailed restriction map of the *ccf* locus. The A46 P-element is located in the 5′ end of the *ccf* transcribed region. NB67 is a full-length cDNA clone corresponding to the long *ccf* mRNA class and NB65 is a full-length clone of the shorter class. No intron was detected by comparison of detailed restriction maps of the cDNAs and the corresponding genomic transcribed region. Molecular analyses of ccf^{420}, a lethal amorphic mutant generated by imprecise excision of the A46 element, revealed a unilateral deficiency of 16 kb, which deleted the entire *ccf* transcribed region. A P-element construct containing a 9 kb *Bam*HI fragment was able to rescue ccf^{420} lethality. (**C**) Developmental profile of *ccf* expression. Two *ccf* transcripts of 3.2 and 3.6 kb are detected by Northern blotting, defining the two cDNA classes. Their expression is high during embryogenesis and peaks at late larval/pupal stages. Note the strong expression of the maternally derived 3.2 kb messenger in 0–1 h 30 embryos. The blot was probed with *rp49* as a loading control.

Sequence analysis reveals a novel protein with an unusual amino acid composition

The NB67 cDNA clone, which was recovered from the 4–8 h library, was 3.5 kb long and was therefore considered to be nearly full-length, and it was sequenced (Figure 2A). In addition, both wild-type and A46 genomic sequences corresponding to the 5′ end of *ccf* were determined, which allowed us to show that the insertion site for the P element lay 18 bp upstream from the 5′ end of the NB67 clone. A putative TATA box was found upstream of the 5′ end of NB67 and of the A46 insertion site. A second TATA box, preceded by a canonical CAAT element, was found 350 bp downstream of the first (see Figure 2A). The spacing of these two TATA boxes is in good agreement with the difference in size between the two classes of cDNA clones analyzed.

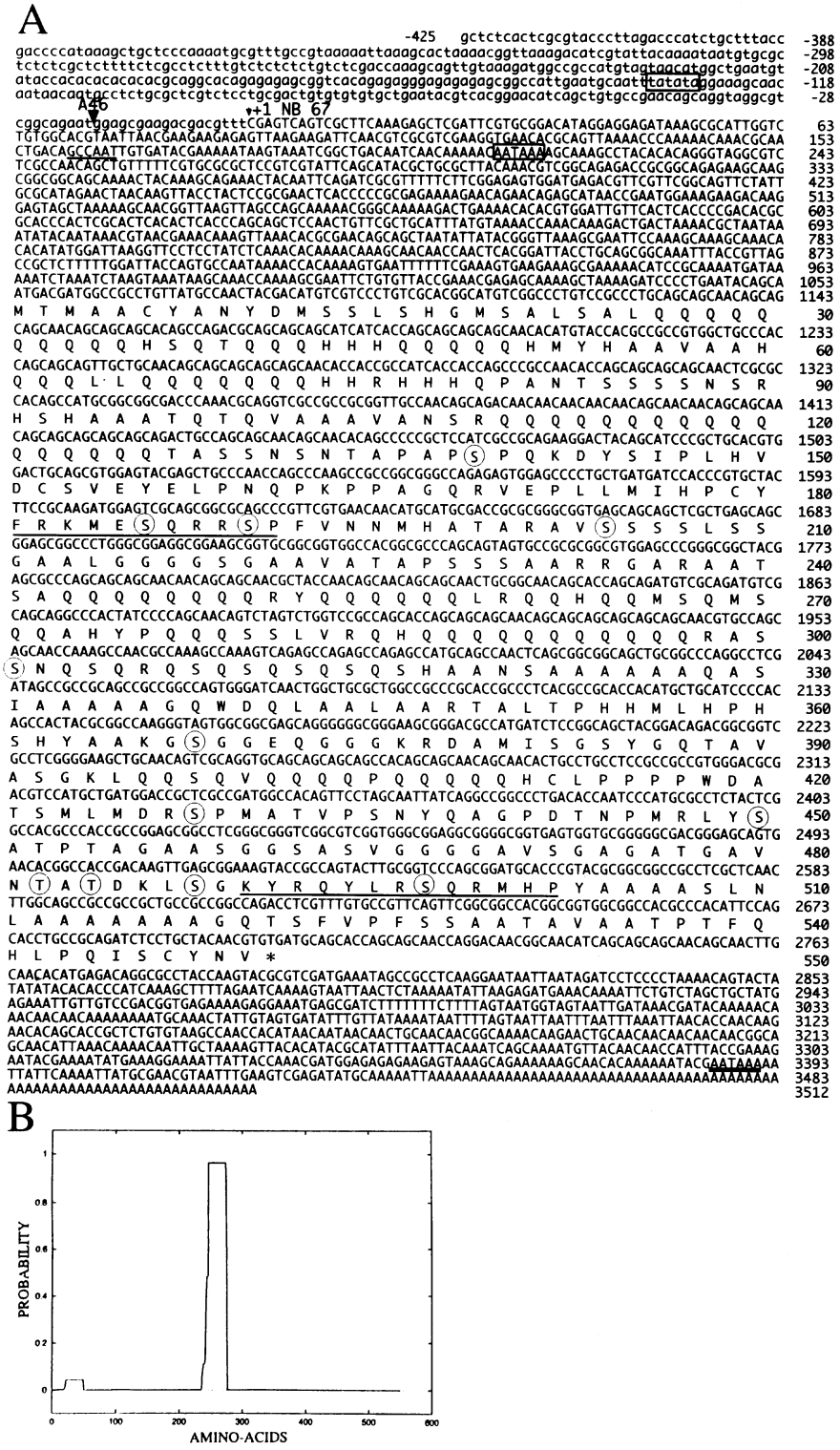

Fig. 2. Nucleotide and predicted amino acid sequence of *ccf*. (**A**) +1 refers to the first nucleotide of NB67 cDNA. The 5′ upstream genomic sequence is given negative numbers and lower cases letters. TATA boxes for the two *ccf* mRNAs are boxed. Note that the A46 insertion site lies downstream from the first TATA box. A consensus CAAT element upstream from the second TATA box is underlined. The unique polyadenylation signal is double underlined. Conceptual translation gives a polypeptide of 550 amino acids. Sequences of peptides designed for antibody production are underlined (P10: amino acids 181–191; P11: 490–502). Encircled residues are potential phosphorylation sites for the $p34^{cdc2}$ kinase (139, 190, 428), S6 kinase II (204, 301, 450), casein kinase II (368, 482) and protein kinase C (186, 484, 488). (**B**) The probability that each residue of CCF will participate in a coiled-coil structure is represented in a bar graph, as calculated by the algorithm of Lupas *et al.* (1991), and shows a peak for region 227–275. The nucleic acid and protein sequences of *ccf* are available in DDBJ/EMBL Genbank and Swissprot databases under the accession Nos U35074 and P41046, respectively.

A 1650 bp putative open reading frame (ORF) encoding a 550 amino acid protein was found, which scored highly for *Drosophila* codon usage. This ORF is preceded by a 1050 bp 5′-untranslated region containing five AUG codons, none of which gave rise to a significant ORF. By contrast, four AUG were found in the first 19 codons of the longer ORF. Since none of these lay in a good context for a *Drosophila* translation start site (Cavener, 1987), we started the conceptual translation from the first one. This potential translation start site is preceded by numerous stop codons in the three frames, further supporting our choice.

No significant similarity of the putative CCF product to any known protein was revealed from database searches. The major feature of the predicted protein is the presence of numerous homopolymeric runs (including polyglutamine, polyalanine, polyserine, polyhistidine, polyglycine and polyproline), separating modules of more balanced amino acid composition. The polyglutamine and polyhistidine runs are encoded by the opa (or M) repeat, frequently encountered in *Drosophila* proteins and especially in nuclear proteins. In the CCF predicted protein, Gln, Ala and Ser represent >50% of the total amino acid content, instead of 20% on average in *Drosophila* proteins, and Gln alone accounts for 25% of the CCF amino acid content. The predicted product of 59 kDa is strongly hydrophilic and shows a basic character (pI = 9.95). In addition, there are three potential $p34^{cdc2}$ phosphorylation sites, three sites for S6 kinase II, two sites for casein kinase II and four sites for protein kinase C. Finally, a short part of the protein, located between amino acids 227 and 275, shows a high propensity to adopt a coiled-coil structure (Figure 2B). Such domains, based on hydrophobic heptad repeats, are thought to be involved in homophilic as well as heterophilic oligomerization (Lupas *et al.*, 1991).

The ccf gene encodes a single 68 kDa product which is associated with mitotic centrosomes and chromosomes

We developed two complementary strategies to raise antibodies against the CCF protein. The first one was to design a fusion protein between β-galactosidase and a CCF polypeptide encompassing amino acids 154–550. This construct allowed us to exclude the first half of polyQ repeats and to retain the major part of the CCF unique regions. Second, in order to circumvent a possible problem of immune cross-reactions due to the homopolymeric runs still present in the fusion protein, we selected from non-repeated hydrophilic regions of the coding region two peptides, P10 and P11, for chemical synthesis (Figure 2A). Polyclonal antisera against all three antigens were raised in rats and tested on Western blots of recombinant β-gal–CCF protein produced in *Escherichia coli*. Antisera directed against both the fusion protein and P11 peptide were positive in this test, whereas antiserum against P10 was not (data not shown). When incubated with Western blots of *Drosophila* embryo protein extracts, both positive antisera recognized a unique polypeptide of 68 kDa, whereas anti-P10 did not give any signal (Figure 3A). The difference between the apparent (68 kDa) and the predicted (59 kDa) protein molecular weights could be due to post-translational modifications, or to the observed biased amino acid content. Saturation experiments with increasing amounts of P11 peptide reduced the reactivity of the anti-P11 antiserum, demonstrating that antibody binding to the 68 kDa antigen was specific (Figure 3B). A Western blot analysis showed that the same 68 kDa polypeptide was detected with anti-P11 (Figure 3C) and anti-fusion (data not shown) antisera at all developmental stages tested, indicating that both *ccf* mRNAs encode an identical product expressed throughout development. When tested in immunohistochemical staining on whole-mount embryos, only the anti-fusion antiserum proved to be positive and was therefore used for further analyses.

Although experiments reported above already argue in favor of the specificity of these antisera, two supplementary lines of evidence strongly reinforce this conclusion. We first took advantage of the existence of a *ccf* deletion mutant [Df(3R)6.7, see below] to check for the absence of antibody reaction. When incubated on Western blots of mutant embryonic extracts, the anti-CCF antibodies did not reveal any difference in the amount of 68 kDa protein as compared with wild-type controls, due both to maternal contribution and the presence of heterozygotes in the progeny (data not shown). However, differences were observed between homozygous and heterozygous mutant embryos upon immunohistochemical analyses: using limiting amounts of anti-fusion antibodies, a quarter of the embryos were devoid of any signal, whereas the remaining part exhibited a weak striped pattern corresponding to the zygotic CCF expression (L.Kodjabachian, in preparation). At higher antibody concentrations, all embryos appeared evenly stained, due to the homogeneous maternal contribution which obscured small concentration variations brought about by the zygotic contribution. Second, we used a heat shock-inducible transgene allowing us to express *ccf* ectopically in order to test the antibody reaction on the induced protein on whole-mount embryos. This experiment revealed the presence of large amounts of CCF in the endoderm, a place where it normally is not detected (data not shown). Taken altogether, these data demonstrated that the anti-β-gal–CCF antiserum is specific and able to detect the CCF protein, both on blots and in embryos.

We then used the anti-fusion protein antibodies to investigate the subcellular location of the CCF protein and found that it was nuclear, at all developmental stages examined (data not shown). Most interestingly, we observed that the CCF protein was still present during cell division, and our results argue for its association with chromosomes and centrosomes as soon as the cells enter mitosis (Figure 4). In syncitial embryos, where rapid successive metachrone mitoses occur until cellularization, the CCF antigen appeared to be associated with condensing chromosomes at early prophase (Figure 4A). At the onset of metaphase, a centrosomal staining appeared which remained detectable until late telophase (Figure 4B and C). During metaphase and anaphase, the CCF staining continuously followed the chromosomes (Figure 4B), while in telophase the signal decreased and spread throughout the reassembled nucleus (Figure 4C). CCF protein showed the same kind of distribution in older embryos (Figure 4D) or imaginal tissues (data not shown). The centrosomal localization of CCF was confirmed by the use of fluorescent double staining with anti-CCF antibody and either Rb188 antiserum, specific for the CP190 centro-

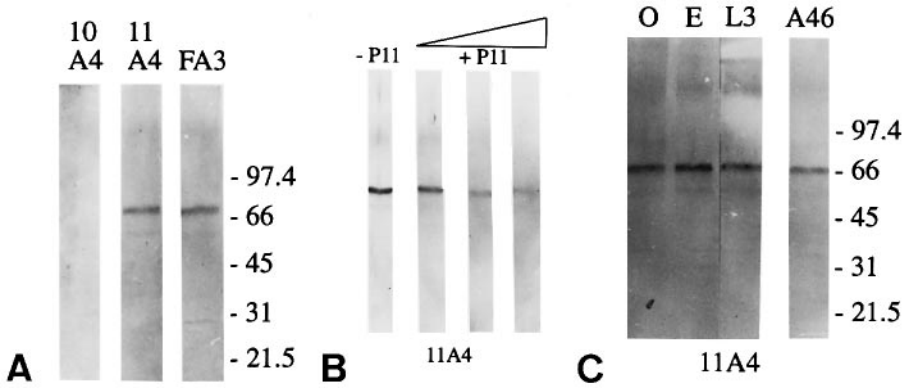

Fig. 3. The *ccf* gene encodes a single product of 68 kDa. (**A**) Crude protein extracts from *Drosophila* embryos probed with anti-P11 (11A4) and anti-fusion (FA3) antibodies revealed a 68 kDa polypeptide whereas those probed with anti-P10 (10A4) antibody did not. (**B**) Antibody saturation experiment with increasing amounts of P11 peptide (50, 100 and 200 μg) demonstrated the specificity of the 11A4 response on *Drosophila* embryonic extracts. (**C**) Developmental Western blot probed with 11A4. The same product was detected in extracts from ovaries (O), embryos (E), third instar larvae (L3) and A46 third instar larvae. Molecular size markers are in kilodaltons.

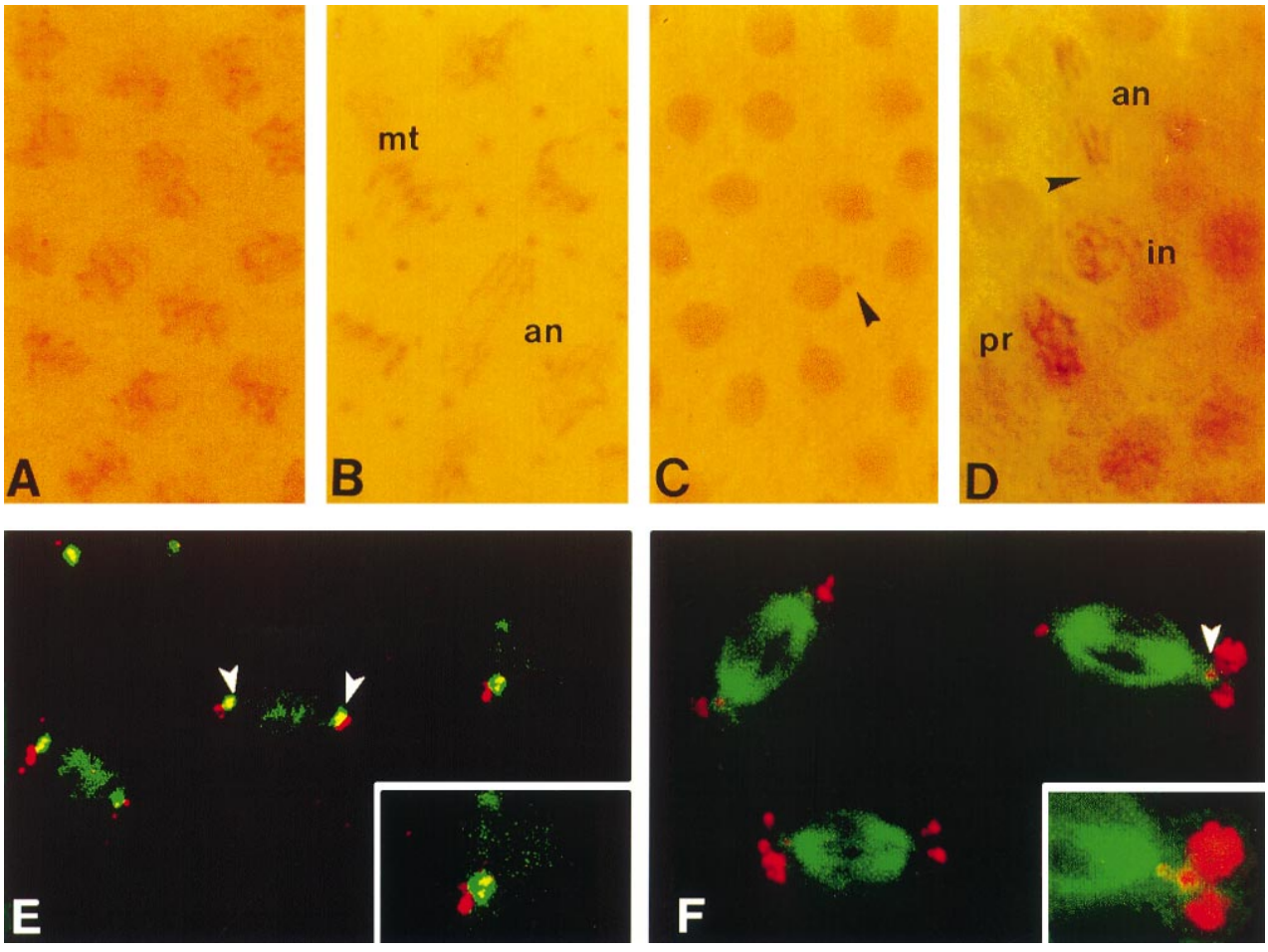

Fig. 4. Subcellular location of the CCF protein during mitotic cycles. (**A**) During prophase (pr) in syncitial embryos, the CCF staining is associated with condensing chromosomes. (**B**) A metaphase (mt) and anaphase (an) field in a syncitial embryo, showing that chromosomes still exhibit the CCF staining during their migration. Note that centrosomes are also strongly labeled (arrowheads). (**C**) At late telophase stage, in a syncitial embryo, the chromosomal staining decreases as the chromatin decondenses and the nucleus reassembles. The centrosomal staining is still obvious (arrowheads). (**D**) A field of mitotic figures in a gastrulating embryo shows that the CCF staining is qualitatively unchanged after cellularization. (**E**) Double staining of a metaphase field in a syncitial embryo, using Rb188 antiserum specific for centrosomal protein CP190 (green) and anti-CCF serum (red). Centrosomal localization is detected as yellow spots (arrowheads). Note that CCF is also present in the pericentrosomal area. (**F**) Double staining of an anaphase in a syncitial embryo using anti-α-tubulin monoclonal antibody (green) and anti-CCF serum (red). Note that at this stage, the pericentrosomal labeling is enhanced and exhibits a punctated appearance. Inserts in (E) and (F) show enlargements of polar regions of the mitotic spindles; embryos corresponding to these panels have been prepared without formaldehyde fixation.

somal protein (Whitfield *et al.*, 1988), or anti α-tubulin monoclonal antibody. The CCF signal clearly co-localizes with the CP190 centrosomal labeling, in which two dots looking very much like the centrioles are visible (Figure 4E, arrowheads and insert). However, the CCF protein is not totally restricted to the centrosome since some pericentrosomal labeling is also visible (Figure 4E), which becomes even more important as mitosis proceeds (Figure 4F). The α-tubulin labeling used here as a control marker for mitotic spindle and nucleating microtubules reveals a co-localization, with CCF limited to a part of the pericentriolar area (Figure 4F, arrowhead and insert). It is well known that labeling of some mitotic components is highly variable depending on fixation conditions and detection techniques (Kellogg *et al.*, 1995; Endow and Komma, 1996). In our hands also, differences were seen depending on the conditions used, since the CCF pericentrosomal labeling has been observed without formaldehyde fixation and the CCF chromosomal labeling has been observed only upon enzymatic immunostaining and 6% formaldehyde fixation. Consistent with these observations, we also noticed that the Rb188 spindle region labeling in early embryos was always clearly apparent upon enzymatic immunostaining (M.Delaage, unpublished results) and rather weak upon fluorescent immunostaining (Figure 4E). A more accurate analysis of CCF localization during cell division will require the use of some other technique, such as the injection of a tagged CCF–green fluorescent protein (GFP) fusion protein in living embryos.

Mutational analysis is consistent with an essential function of ccf in cell proliferation

Mutations in the *ccf* gene were induced by imprecise excision of the A46 element. Independent excision events were recovered on the basis of loss of the $rosy^{+}$ phenotype and tested for lethality in combination with Df(3R)6.7 (82D/E-82F). Ten alleles showed a weakly reduced viability in hemizygous or homozygous animals (comprising between 10 and 50% of the expected number of individuals). Two alleles (ccf^{420} and ccf^{448}) were genetically defined as amorphic mutants, since lethality occurred at the same time and at the same level in both homozygous and hemizygous individuals. In these mutants, embryogenesis was always accomplished accurately, and lethality spread over the larval and pupal stages until the pharate adult eclosion. Homozygous ccf^{420} individuals developed very slowly as compared with heterozygotes, showed little activity during larval stages and exhibited imaginal discs and brain of severely reduced size (Figure 5B). In addition, we show here (Figure 5C) that the ccf^{420} mutation also limits the size of imaginal discs in *l(2)gl* mutants, suggesting that *ccf* is required for normal and tumorous cell divisions. Salivary glands, ring glands, hematopoietic organs and gastric caeca were also poorly developed in mutant larvae (data not shown). We occasionally observed melanotic masses, attached to salivary glands or gastric caeca, and, more rarely, circulating in body cavities (data not shown). Most of the homozygous or hemizygous individuals which escaped larval and pupal lethality appeared extremely weak, failed to extricate themselves from the puparium and eventually died. About 10% of amorphic mutants survived up to adulthood but exhibited a reduced longevity compared with wild-type animals. These adults showed cuticular defects, such as loss or duplication of external sensory organs, loss of abdominal bristles and aberrant posterior cross-vein formation (data not shown). They also demonstrated a global reduction of structures derived from imaginal discs, such as appendages, head and eyes, whereas the abdominal cuticle was of a regular size (Figure 5D). Both crosses between mutant females and wild-type males and reciprocal crosses were fertile, whereas crosses between mutant individuals led to complete lethality of progeny, spread over embryonic and larval stages.

Molecular analyses of the mutations have shown that ccf^{420} had deleted the entire *ccf* coding region (Figure 1B), confirming that it is a true amorphic allele. Weaker alleles showed no detectable rearrangement in the *ccf* region. A 9 kb genomic DNA fragment containing only the *ccf* transcription unit with flanking sequences (see Figure 1B) was cloned into the pCasper4 vector and introduced into wild-type flies by germline transformation in order to rescue the ccf^{420} mutant. We observed a complete rescue of ccf^{420} homozygotes, since the percentage of surviving adults increased from 23% (n = 1188) to 100% (n = 961) of the expected number, in the presence of the transgene. In addition, the complete lack of viable progeny of *ccf* mutants was also partially corrected in the presence of the transgene, enabling us to maintain homozygous mutant stocks rescued by the transgene. These data indicate that *ccf* inactivation alone is responsible for lethality and for the accompanying phenotypic defects described herein.

ccf is required for mitotic chromosome condensation

Both the cellular localization of the CCF product during mitosis and the developmental defects described above were suggestive of an essential function of *ccf* in the cell cycle. We therefore decided to examine mitosis progression in ccf^{420} mutants, and analyzed aceto-orcein squashes of third instar larval brains. In order to determine mitotic indices and metaphase to anaphase ratios accurately, we avoided treatment with colchicine and hypotonic solution, which are known to block cells in metaphase and to decrease the number of anaphases, respectively. We found that the mitotic index was increased in a ccf^{-} background with respect to wild-type, and that metaphase frequency was greatly increased with respect to anaphase frequency (Table I). This demonstrated that mutant cells were able to enter mitosis but were unable to progress through it. This failure to complete mitoses could arise from either a block in the metaphase to anaphase transition, or a problem in mitotic spindle formation and anaphase progression. Detailed analyses suggested that the CCF protein was involved in condensation of chromosomes at mitosis. Indeed, >80% of mutant metaphases showed poor and irregular chromosome condensation, whereas in a wild-type third instar larval brain only 3% of metaphases were poorly condensed. In *ccf* mutants, chromosomes still appeared as if arranged on a metaphase plate but they failed to condense properly into distinct entities and formed, in the most extreme cases, a chromosomic cloud at the center of the cell (Figure 6B–D). Treatment of *ccf* mutant brains with colchicine and hypotonic solution revealed neither extensive chromosome breakage nor changes in the number of polyploid or aneuploid mitotic

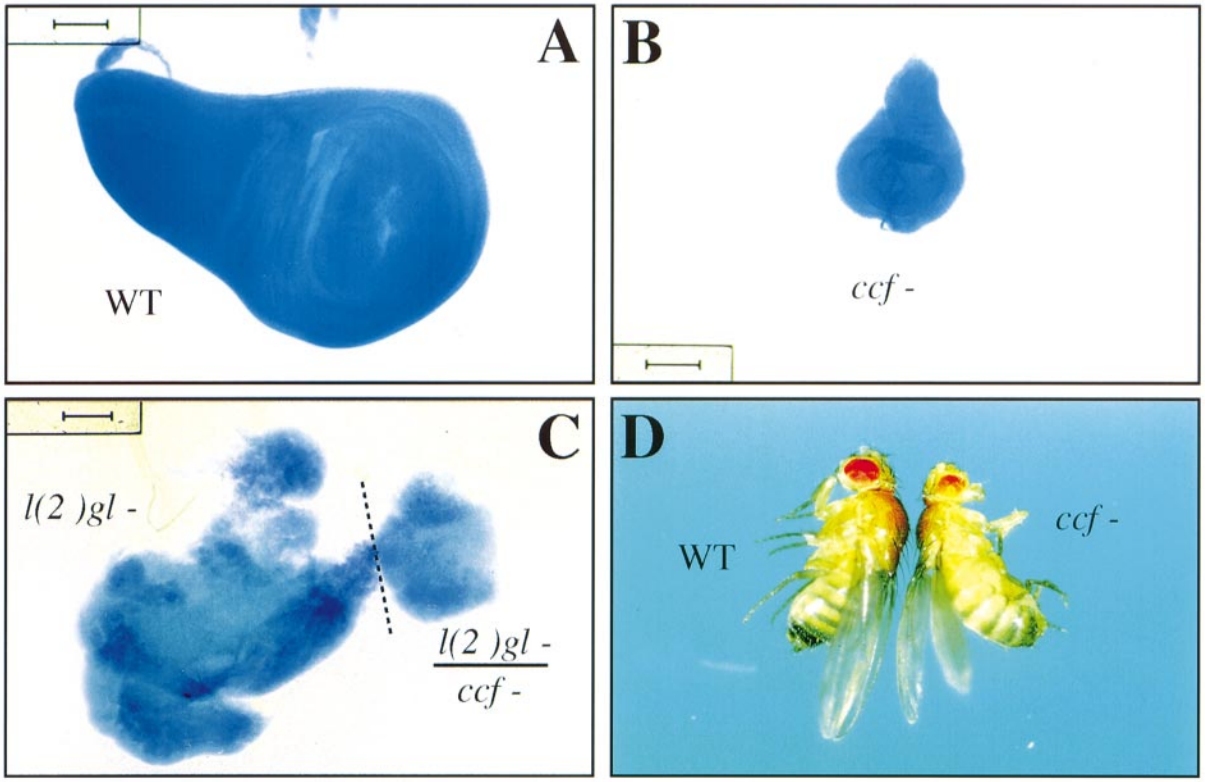

Fig. 5. The 'small disc' phenotype of a null *ccf* mutant. (**A** and **B**) Wild-type and ccf^{420}/ccf^{420} third instar wing discs, respectively. Photographs are shown at the same magnification. Scale bars, 30 μm. (**C**) Third instar wing discs of larvae homozygous for a *l(2)gl* deficiency (left) or doubly homozygous for *l(2)gl* and ccf^{420} (right). Note that despite the loss of epithelium folding which is characteristic of the *l(2)gl* mutation, the size of the tumoral disc is much reduced when the *ccf* function is absent. Imaginal discs in (A–C) were stained with toluidine blue to enhance their contrast. (**D**) Wild-type (left) and ccf^{420}/ccf^{420} (right) adult females. The overall sizes of *ccf* mutant imaginal derivatives are reduced, though they retain their normal morphology.

Table I. Quantification of mitotic activity in ccf^{420}/ccf^{420}, ccf^{420}/Df 6.7 and wild-type third instar larval brains

Genotype	Mitotic[a] figures	Fields[b]	No. of metaphases	No. of anaphases	Mitotic[c] index	Metaphase frequency	Anaphase frequency
WT	567	375	431	136	1.51	0.76	0.24
ccf^{420}/ccf^{420}	758	364	712	46	2.08	0.94	0.06
ccf^{420}/Df 6.7	788	402	701	87	1.96	0.89	0.11

[a]Ten brains were scored for each genotype.
[b]Microscopic area observed under phase-contrast (100×10).
[c]Mitotic index is defined as the ratio of the number of mitotic figures to the number of examined fields.

figures, compared with wild-type chromosomes (data not shown). This last observation indicated that defective mutant cells were not able to reach the following metaphase, giving a clue to the 'small disc' phenotype.

Loss and gain of function of ccf lead to homeotic transformations

Among other phenotypes, adults homozygous for the ccf^{420} allele showed extra sex comb teeth on the second tarsus of the first leg, with a frequency of ~50% (Table II). The appearance of this homeotic phenotype prompted us to test if overexpression of CCF protein could also perturb homeosis in the fly. We used the Gal4/UAS (upstream activating sequences) system of induction (Brand and Perrimon, 1993), in which Gal4 acted as a transcriptional activator upon a UAS-*ccf* transgene and directed the expression of *ccf* according to its own pattern of expression. As a driver, we used *patched*-Gal4 (Hinz *et al.*, 1994), which is expressed along the anterior–posterior border of imaginal discs (not shown). Flies carrying the *patched*-Gal4 driver were crossed with those carrying the UAS-*ccf* construct. Since the efficiency of the Gal4-UAS system is known to be temperature dependent, we mated these flies at 18, 22, 25 or 29°C. We found no major differences in the penetrance of the observed phenotypes, but rather noticed an increased expressivity with higher temperatures. No lethality was observed during embryonic development, although *patched*-Gal4 is expressed during this period. Very little lethality occurred later either, and the majority of individuals differentiated into pharate adults. A small proportion of flies were unable to extricate themselves from the pupal case, but showed the same range of phenotypes as the hatching flies. The most obvious phenotype we observed concerned a systematic

Table II. Genetic interactions between *Pc-G* and *ccf* mutations

	% of T2 leg with sex combs	Average no. of teeth per comb	% of T3 leg with sex combs	Average no. of teeth per comb	% of T1 leg with sex combs on t2	Average no. of teeth per comb
$ccf^{420}/+$	0	0	0	0	0	0
ccf^{420}/ccf^{420}	0	0	0	0	50	1.5
$A46/Pc^{K}$	76	2.2	16	1.75	0	0
ccf^{420}/Pc^{K}	96	4.1	82	2.2	1	1
$A46/Pc^{3}$	63	1.6	1.4	1	0	0
ccf^{420}/Pc^{3}	80	2.6	23	1.7	0	0
$A46;\ ph^{5PS}$	80[a]	5.25	36	4.6	0	0
$ccf^{25};\ ph^{5PS}$	100[b]	9.8	83	5.1	0	0
ph^{410}	100	6	95	4.7	0	0
$ccf^{420}/+;\ ph^{410}$	100	9.5	100	8.1	13	1
$ccf^{25}/+;\ ph^{410}$	100	9.1	100	7.8	10	1
$E(z)^{60}/+$	0	0	0	0	0	0
$ccf^{420}/E(z)^{60}$	21	ND	12	ND	7	ND
$ccf^{448}/E(z)^{60}$	23	ND	10	ND	8	ND

Approximately 50 individuals were examined for each genoytype. ccf^{25} and ccf^{448} are null alleles as defined by complementation tests over Df(3R) 6.7.
[a]6% of these individuals exhibit rotated genitalia.
[b]30% of these individuals exhibit rotated genitalia.
ND, not determined.

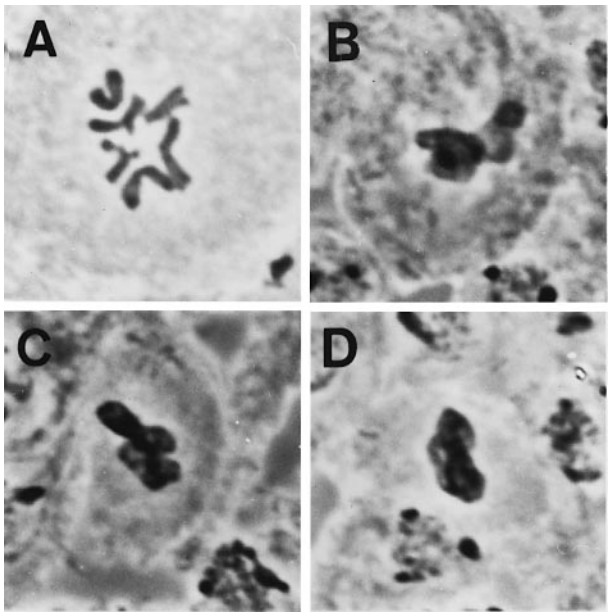

Fig. 6. Chromosome condensation during mitosis is defective in ccf^{-} mutant third instar neuroblasts. (**A**) Wild-type metaphase plate. (**B**–**D**) Metaphase figures from ccf^{-} mutant neuroblasts. These figures show that the ccf^{-} mitotic chromosomes never reach an optimal level of condensation and fail to separate properly from one another.

transformation (100%) of arista toward tarsal segments of the leg (Figure 7). Consistent with this phenotype, *patched*-Gal4 expression is found in the prospective arista in the antennal imaginal disc (data not shown). Most of the transformed legs included the distal-most tarsal structures, namely the tuft and the claw (Figure 7B and C). We also observed fully penetrant weak transformation of the halteres into wings as assessed by the presence of rows of long hairs, rotated genitalia and loss of scutellar bristles (data not shown). Finally, we observed a gradual reduction in the size of legs, wing blades and scutellum, in a temperature-dependent manner. We conclude from these experiments that both loss of function and overexpression of *ccf* dramatically disturb the homeotic program.

ccf mutations interact with Pc-G genes mutations

The additional sex comb found on the first leg of ccf^{420} homozygotes is reminiscent of some Pc-G mutant phenotypes such as *Polycomb* (*Pc*), *polyhomeotic* (*ph*), *Enhancer of zeste* [*E(z)*] and *multi sex combs* (*mxc*) (Santamaria, 1993). This observation prompted us to look for genetic interactions between *ccf* and Pc-G mutations. We found that *ccf* amorphic mutations enhanced the extra sex combs phenotype of several alleles of *Pc*, *ph* and *E(z)*, in a dominant way, both quantitatively and qualitatively (Table II). Interestingly, different aspects of the phenotype of Pc-G mutations used here were enhanced. For instance, we observed an increased rate of rotated genitalia in flies carrying ph^{5PS} and ccf^{25} (Table II). We also observed that the extra sex combs phenotype on the second tarsus of the first leg was enhanced in genetic combinations including *ccf* and *ph* mutations and, more interestingly, *ccf* and *E(z)* mutations (Table II). As heterozygous *E(z)* mutations never exhibit this phenotype (Jones and Gelbart, 1990), this observation strengthens the idea of a dominant interaction with *ccf*.

The CCF and the Posterior sex combs (Psc) Pc-G proteins share common sites on polytene chromosomes

Since Pc-G proteins recognize a discrete number of common specific sites on polytene chromosomes, the interaction described above between *ccf* and Pc-G genes prompted us to test if the CCF protein was also able to bind to polytene chromosomes. For this purpose, salivary gland chromosome squashes were prepared and incubated with anti-β-gal–CCF antibodies. The results clearly show that CCF binds to polytene chromosomes where it specifically and reproducibly recognizes >100 sites with variable intensity (Figure 8A). We then decided to compare

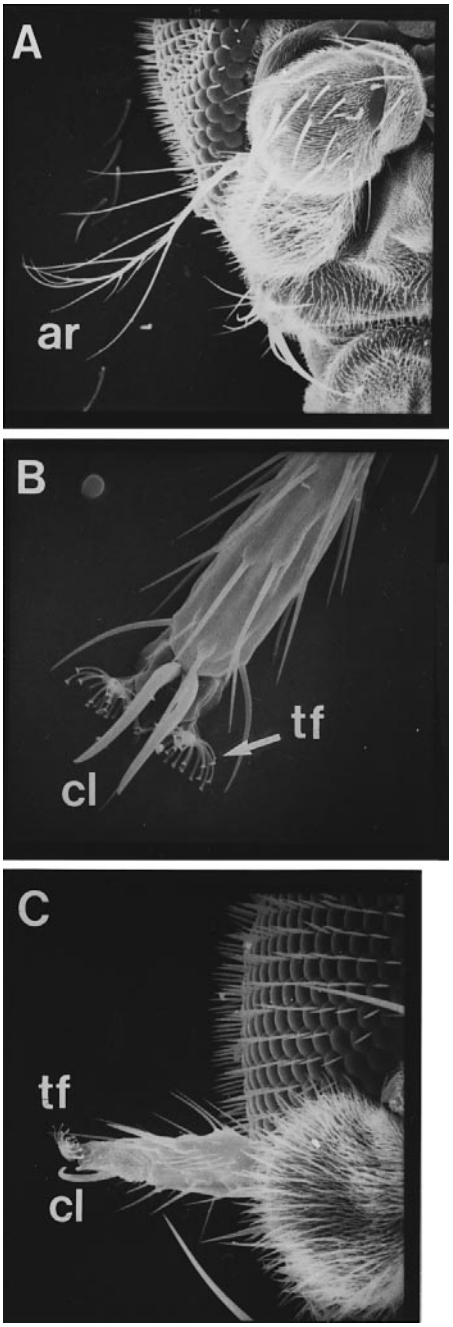

Fig. 7. Aristapedia phenotype due to overexpression of CCF. (**A**) Wild-type antennal segment including arista (ar). (**B**) Wild-type leg showing the distal-most aspects of the fifth tarsal segment, the claws (cl) and the tufts (tf). (**C**) Antennal segment of an individual containing *ptc*-Gal4 and UAS-*ccf* constructs. Note the transformation of the arista toward a half-leg including aspects of the last tarsal segment.

directly the CCF binding sites distribution pattern with that of one member of the Pc-G proteins and, for that purpose, we used anti-CCF and anti-Psc antibodies in double labeling experiments. It is clear from the results presented in Figure 8B that these two proteins share a great number of binding sites on polytene chromosomes. We estimated from several spread nuclei that a majority of the Psc sites were also recognized by the CCF protein. Some sites, however, seemed to be unique to either the Psc (Figure 8B and D) or the CCF proteins.

Taken together with the genetic interaction data, these results suggest that functions of the Pc-G genes and that of *ccf* are indeed related and that their products share several common target genes.

Discussion

The ccf gene encodes a novel product

Sequence analyses define CCF as a novel protein, since no significant similarity was found with any reported product. The most striking features of the sequence are the presence of several homopolymeric runs and the high isoelectric point, which might reveal an affinity of CCF for nucleic acids. In addition, a short region in CCF displays a high probability of forming a coiled-coil structure. Such domains potentially are involved in protein–protein interactions and frequently are encountered in polymerizing proteins such as keratin, tubulin and myosin, in oligomerized complexes such as G-protein β-subunits, and in leucine zipper transcriptional activators. Interestingly, large coiled-coil domains exist in the motor proteins of the SMC family which are required for chromosome condensation (reviewed by Hirano *et al.*, 1995). Several putative phosphorylation sites, including notably consensus sites for S6 kinase II and $p34^{cdc2}$, which are potent mitotic kinases (Ward and Kirschner, 1990; Dorée and Galas, 1994), are found in the CCF sequence. Post-translational maturation by phosphorylation could explain the increase in the apparent size (68 kDa) with respect to the predicted size (59 kDa); this would be in good agreement with a mitotic role for the CCF product, since entry into mitosis is dependent on a phosphorylation cascade promoted by the cdc2 kinase (Dumphy, 1994).

What is the role of CCF during mitosis?

The CCF protein is detected during embryonic development and in proliferating tissues during larval development and oogenesis. Loss-of-function mutations in *ccf* give rise to developmental defects in imaginal discs and the central nervous system of third instar larvae, resulting in a massive, but not total, death of mutant individuals at late larval and pupal stages. This delayed lethality could be explained by the maternal supply allowing embryogenesis to be completed in the absence of zygotic product, as is the case for the third class of cell cycle mutants (Gatti and Baker, 1989). Indeed, maternally derived CCF mRNA and protein are detected in syncitial embryos. Another argument in favor of a maternal contribution of the CCF protein to development comes from the difference in the survival rate of ccf^{420} homozygous mutants, depending on the genetic cross from which they are issued. The fact that crosses between homozygous mutant escapers never give rise to a viable progeny, whereas 10% of homozygous individuals issued from a cross between ccf^{420} heterozygotes are able to survive, indicates that the CCF maternal contribution can rescue the defective zygotic development somewhat. However, the fact that homozygous mutant females crossed with wild-type males are fertile indicates that embryonic mitoses occurring before cellularization are not critically dependent on maternal CCF.

CCF seems to be associated with condensing mitotic chromosomes as soon as cells enter mitosis and during the subsequent steps of the mitotic cycle. Moreover, we

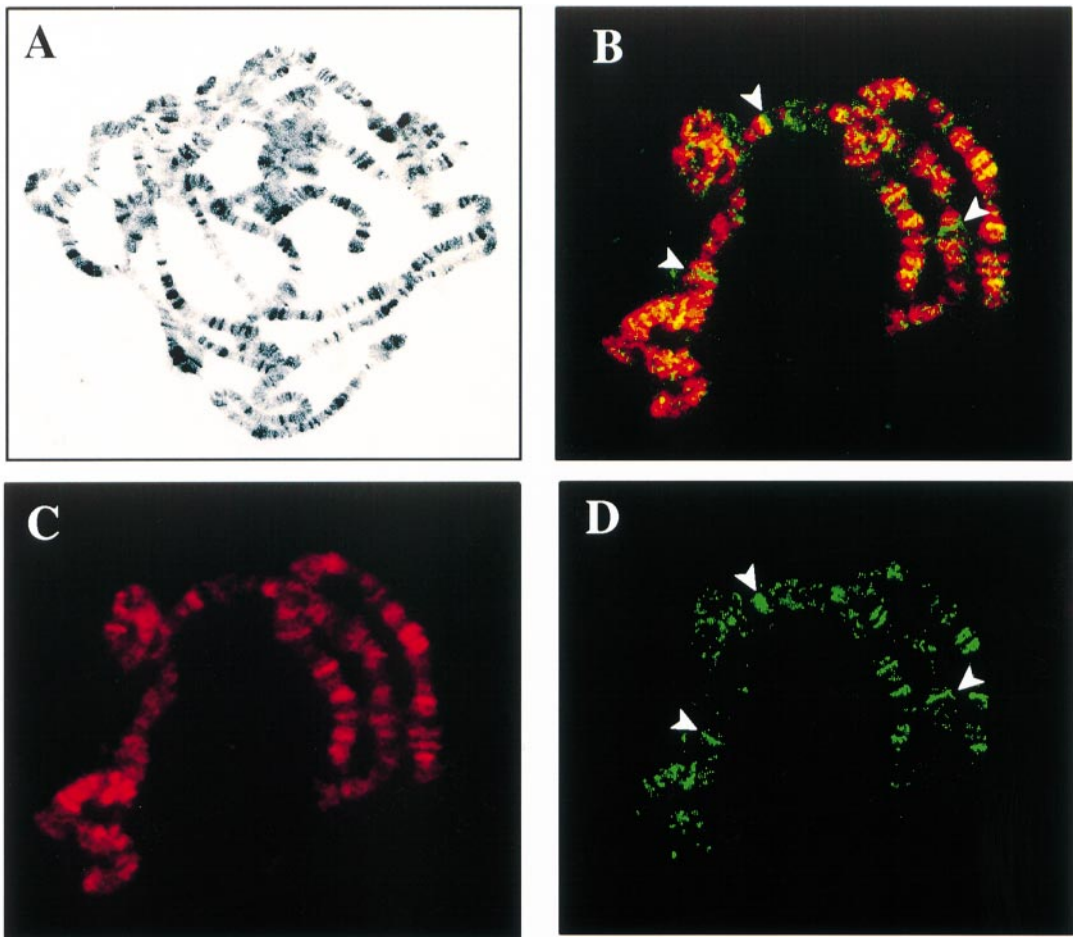

Fig. 8. Immunohistochemical localization of CCF protein on polytene chromosomes and relationship to Psc protein localization. (**A**) Polytene chromosome staining with anti-CCF antibody showing multiple binding sites. (**B**) Fluorescence immunolocalization of both CCF (red) and Psc (green) proteins on the same chromosome regions. Note that both proteins co-localize at many sites (yellow). (C and D) Unmerged fluorescence immunolocalizations of CCF (**C**) and of Psc (**D**) proteins corresponding to (B). Arrowheads in (B) and (D) point towards Psc unique sites.

noticed a large number of chromosome condensation defects in mutant larval brains. Altogether, these data could indicate a role for CCF in chromosome condensation. XCAP-C and XCAP-E, two members of the SMC family of proteins, are associated specifically with mitotic chromosomes and not with interphase chromatin (Hirano and Mitchison, 1994). It has been proposed, as a possible model, that this reversible association could be mediated by a mitosis-specific chromatin receptor (Peterson, 1994). CCF could be such a molecule, serving to anchor SMC complexes or any other component on chromatin during the course of mitosis. Alternatively, CCF could be a direct effector of chromosome condensation.

During cell division, CCF staining seems also to be associated with the centrosome and the pericentrosomal region through metaphase to anaphase stages. This localization could suggest a role for CCF in centrosome function, a possibility which is of general interest since, to date, no other antigen has been found associated with both centrosome and chromosome arms during mitosis in eukaryotic cells (for review, see Kalt and Schliwa, 1993). The centrosome serves to anchor growing microtubules and thereby takes part in the shape, polarity and internal order of eukaryotic cells. Upon entry into mitosis, spindle assembly is accompanied by a 5- to 10-fold increase in microtubule nucleating activity of the centrosome. This process parallels the relocalization of many 'passengers' on the centrosome and especially that of a small fraction of the Mitosis Promoting Factor (reviewed by Kellogg *et al.*, 1994). In *Drosophila*, CCF appears to be one of these mitosis-specific centrosomal factors. We found a marked reduction of anaphase frequency in *ccf* mutant brains, which indicates that mitosis fails to progress correctly. This delayed entry into anaphase could be due to defects in spindle assembly at the level of the centrosome. Alternatively, it could result from the lack of condensation of chromosomes, which in turn could fail to bind the mitotic spindle properly. Further examination of mitotic spindle structure in *ccf* mutants should help to investigate a putative role for this factor in centrosome function during mitosis.

Finally, and as is discussed later on, CCF is found associated with several discrete sites on polytene chromosomes. It is intriguing to note that CP190, another mitosis-specific centrosomal factor, is also found associated with specific sites on polytene chromosomes (Whitfield *et al.*, 1995). It would be interesting to test if other centrosomal proteins are also able to bind specifically to polytene chromosomes.

Is ccf involved in epigenetic control of gene expression together with Pc-G genes?

We found that *ccf* mutations produced an extra sex combs phenotype and also enhanced the same kind of phenotype from mutations of Pc-G genes. Moreover, we have shown that overexpression of CCF can induce homeotic transformations in the adult fly. However, one could note an apparent discrepancy between loss-of-function and gain-of-function homeotic phenotypes of *ccf*. Indeed, while amorphic *ccf* mutants exhibit Pc-G-like phenotypes, part of the phenotypes due to overexpression of CCF are rather trx-G-like (i.e. the transformation of arista into tarsus and the Ubx-like phenotype of halteres). An interesting parallel can be traced to work by Brunk and Adler (1990), who studied a mutation called *Aristapedioid*, which provoked phenotypes similar to some of those exhibited by CCF-overexpressing animals, namely transformation of arista into tarsus and loss of scutellar bristles. These authors have shown that this mutation was a dominant gain-of-function allele in the *Suppressor 2 of zeste* gene, an enhancer of Pc-G genes (Brunk and Adler, 1990). Moreover, they showed that directed overexpression of this gene or its close relative *Posterior sex combs*, a member of the Pc-G, was sufficient to mimic the observed *Aristapedioid* phenotypes (Sharp *et al.*, 1994). More recently, LaJeunesse and Shearn (1996) demonstrated differential requirements of the E(z) protein as a Pc-G or a trx-G factor, depending on spatial and temporal cues, as well as on the homeotic target gene locus examined. These different studies illustrate how the developmental consequences of the deregulation of a Pc-G gene are not necessarily predictable, and rather depend on the combinations and respective amounts, at a given site, of the Pc-G and trx-G proteins. This could apply for *ccf* as well, as a dominant enhancer of Pc-G genes.

The enhancement of Pc-G mutations by *ccf* mutations in trans-heterozygotes argues strongly for a direct interaction between these gene products on the chromatin. The CCF protein is present in the nucleus of interphasic cells and, furthermore, is associated with polytene chromosomes at a number of discrete loci, many of which are shared with the Psc protein. These results suggest that *ccf* and Pc-G genes, which exhibit interactions at the genetic level and share several possible common target genes at the molecular level, are functionally related.

Are there factors involved in chromatin structure during both mitosis and interphase?

The *ccf* gene is necessary for progression through mitosis and provokes homeotic phenotypes upon deregulation. In both of these apparently unrelated phenomena, CCF might act at the level of the chromatin organization. Recent studies have demonstrated that some factors involved in heterochromatin formation at interphase are also essential for structuration of the chromosome and its segregation during mitosis. *Drosophila* heterochromatin protein 1 (HP1), which shares a functional domain with the Polycomb protein, is necessary for chromosome condensation during syncitial mitoses (Kellum and Alberts, 1995). Most interestingly, the GAGA factor, which is encoded in the fly by the gene *Trithorax-like*, a member of the trx-G (Farkas *et al.*, 1994), is also required for correct chromosome segregation at mitosis (Bhat *et al.*, 1996). It is also interesting to mention here that the chromosomal protein Psc is associated with condensed chromosomes during syncitial mitoses (Martin and Adler, 1993), suggesting that this factor could be required to maintain the chromosome organization during cell division. Furthermore, one must underline that the Pc-G gene *E(z)*, which was first identified through a cell cycle mutation showing a small disc phenotype, is required to maintain chromosome integrity during both mitosis (Gatti and Baker, 1989) and interphase (Rastelli *et al.*, 1993). Here, we show that *ccf* shares many phenotypic characters with *E(z)* and is able to enhance its homeotic phenotypes. This raises the question of a potential role for other Pc-G genes during cell division. The silencing of targeted loci established by Pc-G proteins is stably inherited through multiple rounds of cell division, and this certainly requires the maintenance of the chromosomal topology during DNA replication and condensation at mitosis. Proteins like E(z) or CCF could participate in this process of maintenance, and their mutations could therefore affect both mitosis progression and silencing dependent on Pc-G factors.

Materials and methods

Drosophila strains and manipulations

Wild-type strain was Oregon R. A46 was P(*lac*$^{+}$, *ry*$^{+}$), *kar*^{1}, *ry*^{506}/TM3, *Sb*, *ry*^{RK}. Jump-start were induced with the *w*^{1118}; *Sp*/CyO; Δ2–3 *Sb*, *ry*^{506}/TM6 stock. *rosy* $^{-}$ revertants were recovered by crossing with *st*, *ry*, *e*/TM3, *Sb*, *ry* and subsequently tested for lethality in combination with Df(3R) 6.7/TM3, *Sb* (Letsou *et al.*, 1991). As the presence of the *ry*^{506} mutation was found to be deleterious in homozygous mutants, we removed it by recombination over a *rosy*$^{+}$ chromosome carrying the recessive markers *red* and *ebony*. The *l(2)gl* mutation used was *l(2)gl*^{4}, a null allele included in a terminal deficiency of the 2L chromosome. *Pc*^{3} is a homozygous lethal gain-of-function allele of *Polycomb*; *ph*^{410} is a homozygous viable allele of *polyhomeotic*; *E(z)*^{60} is a homozygous lethal antimorphic allele of *Enhancer of zeste* (Lindsley and Zimm, 1992). *Pc*^{K} is a strong hypomorphic mutation of *Polycomb* (R.Paro, personal communication), and *ph*^{5PS} is a strong hypomorphic viable allele of *polyhomeotic* (P.Santamaria, personal communication).

The rescuing construct was obtained by cloning a 9 kb *Bam*HI fragment spanning the *ccf* transcription unit into a pCasper4 vector. HS-*ccf* and UAS-*ccf* were generated as follows: the 5′-most 3 kb *Hin*dIII fragment of cDNA clone NB67 was subcloned into pBluescript, which enabled us to recover an *Eco*RI fragment extending from positions +1006 to +2871 for insertion into the pCaSpeR-hs or the pUAST vectors, respectively. These constructs encompass the whole CCF predicted coding region. Germline transformation was performed as described in De Zulueta *et al.* (1994).

Aceto-orcein squashes of third instar larval brain were prepared as described in Karess and Glover (1989).

Sequence determination and analysis

Several cDNA clones were recovered from Nick Brown 0–4 h and 4–8 h libraries. The longest clone, NB67 (3.5 kb), was subcloned in both orientations into pKS^{+}, and deletion series were made as described in Henikoff (1987). Sequencing was performed on single-stranded DNA on both strands using the double-stranded chain termination method (Sanger *et al.*, 1977). The sequencing project was developed with the Geneworks program (Intelligenetics) on a Macintosh computer (Apple Inc.). Sequence similarity searches were performed at NCBI through InterNet, using blastp and tblastn programs (with the SEG+XNU filtering option to eliminate the contribution of homopolymeric runs) on nr (non-redundant) and dbest databases.

Antibody production and immunohistochemistry

The β-gal–CCF fusion construct was generated by insertion of a *Pst*I–*Pst*I fragment from pKS-NB67 extending from position +1513 to +2871 into the pEx3 vector (Stanley and Luzio, 1984). This encodes a recombinant β-gal–CCF protein, comprising amino acids 154–550 of CCF placed at the C-terminus of β-gal. The pop2136 bacterial strain

was used for transformation, and the hybrid protein was recovered as a non-soluble product and extracted from a 5% preparative SDS–polyacrylamide gel.

The P10 and P11 peptides were designed with the following criteria: they should be ~15 amino acids long, be derived from hydrophilic regions free of proline and glycine, and exclude any homopolymeric run. A Kyte and Doolittle graph showed that the CCF protein was highly hydrophilic, but a detailed analysis revealed only three convenient regions to define a peptide fitting all criteria. P10 peptide is YAAFRK-MESQRRSPF, where YAA has been added as a spacer arm; P11 peptide is AAAYRQYLRSQRMHPYA, AAA also being a spacer arm (Figure 3A). Peptide synthesis was carried out by Immunotech, Marseille. Both peptides were coupled to bovine serum albumin (BSA; Sigma) as a carrier protein at a 30:1 ratio.

Rats (Lou strain) were immunized with 100–150 μg of each antigen, and boosts were spaced 4 weeks apart. Immune sera were recovered 10 days after the third boost for anti-fusion sera and 10 days after the fourth boost for anti-peptide sera. Pre-immune sera did not react with *Drosophila* proteins. Anti-fusion sera were cleared from anti-*E.coli* and anti-β-gal antibodies by incubation with an acetone powder prepared from an induced culture of pop2136 transformed with an empty pEx vector. Test experiments were also performed using anti-CCF antibodies which had been affinity purified on β-gal–CCF recombinant protein blotted onto nitrocellulose according to Sambrook *et al.* (1989). Since we found no qualitative differences between affinity-purified and cleared antibodies, we omitted the purification in the reported experiments.

Immunohistochemical analysis on polytene chromosomes was performed according to the method of Zink and Paro (1989). For whole-mount immunodetection, embryos were dechorionated in 50% bleach and fixed for 12 min in 1:1 100 mM PIPES (pH 6.9), 1 mM $MgCl_2$, 1 mM EGTA, 6% formaldehyde/heptane; formaldehyde was omitted when methanol fixation alone was needed. Devitellinization was performed in 1:1 95% methanol, 25 mM EGTA/heptane. Devitellinized embryos were rehydrated sequentially in methanol/phosphate-buffered saline (PBS) (80/20, 60/40, 20/80) then in PBS, and saturated in PBS, 0.1% Triton, 5% low fat dry milk for 1 h at room temparature. Pre-cleared anti-fusion antibodies or affinity-purified antibodies were pre-incubated at a dilution of 1:10 on formaldehyde- or methanol-fixed embryos and used at a final dilution of 1:500 overnight at 4°C in the saturation buffer. For immunoenzymatic staining, detection was performed using the Vectastain Elite kit (Vector Laboratories). Microscopic observations were made with a Zeiss Axiophot microscope equipped with Nomarski optics. For immunofluorescent staining, donkey tetramethylrhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated anti-rat or anti-mouse secondary antibodies were obtained from Jackson immunoResearch Labs, Inc. Confocal microscopy was carried out using a ZEISS LSM 410.

Acknowledgements

We thank C.O'Kane for the gift of the original A46 enhancer trap line, S.Wasserman for providing deficiencies in region 82E, J.M.Dura for the anti-Psc antibody and P.Santamaria and J.Deutsch for providing Pc-G mutants. We are grateful to S.Kerridge, M.Semeriva, R.Karess, P.Santamaria and J.Deutsch for useful discussions during the course of this work. We thank J.Pradel, C.Goridis and M.A.O'Reilly for their critical reading of the manuscript and the anonymous referees for their very insightful comments. This work was supported by the CNRS, grants from l'Association pour la Recherche contre le Cancer to J.Pradel and doctoral fellowships from MESR to C.M. and L.K. and from l'Association pour la Recherche contre le Cancer to L.K.

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Received February 11, 1997; revised November 25, 1997; accepted December 3, 1997