Development 124, 3385-3394 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV2189

The *cramped* gene of *Drosophila* is a member of the *Polycomb*-group, and interacts with *mus209*, the gene encoding Proliferating Cell Nuclear Antigen

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SUMMARY

We have isolated and molecularly characterized the *cramped* (*crm*) gene of *Drosophila melanogaster*, and show that it can be classified as a *Polycomb*-group (*Pc*-G) gene. *crm* mutants exhibit typical *Pc*-G mutant phenotypes, reminiscent of ectopic homeotic gene expression, with additional sex comb teeth found on mesothoracic and metathoracic legs, and proximodistal transformations of the tarsal segments.

***crm* encodes an 693 amino acids protein, with no significant homology to known proteins. We used polyclonal antibodies raised against bacterially expressed truncated CRM protein to show that the *crm* gene product is localized to the nucleus during embryogenesis. This nuclear localization appears to be restricted to S-phase nuclei, as CRM immunostaining disappears at mitosis. We found that this cell-cycle-dependent staining pattern was identical to that of Proliferating Cell Nuclear Antigen (PCNA). Furthermore, we provide evidence for co-localization of CRM and PCNA proteins in salivary gland polytene nuclei, and for a genetic interaction between *crm* and *mus209*, the *Drosophila* gene encoding PCNA. Together, our data suggest that these two proteins are involved in a common regulatory pathway and highlight possible interactions between *Pc*-G-mediated silencing and DNA replication in *Drosophila*.**

Key words: *Polycomb*-group, *cramped*, *Drosophila*, silencing, PCNA, *mus209*

INTRODUCTION

The homeotic genes of *Drosophila* are involved in specifying segmental identity along the anteroposterior (A/P) axis. These selector genes are clustered at two large genomic loci. Genes in the *Antennapedia* complex (ANT-C) are responsible for the identity of the head and the two first thoracic segments, and genes in the *Bithorax* complex (BX-C) determine the identity of the third thoracic and the eight abdominal segments (reviewed in McGinnis and Krumlauf, 1992; Gehring et al., 1994). Each homeotic protein is expressed in a precise domain along the A/P axis. This complex pattern of homeotic gene expression is established early in development in part by the gap and pair-rule gene products. As these transcriptional regulators are expressed only early and transiently, a maintenance mechanism has to be set up to ensure correct homeotic gene expression throughout development. The mechanism responsible for maintenance involves transcriptional regulators, which can be subdivided into two groups: genes of the *trithorax*-group (*trx*-G), which are needed to maintain homeotic genes active in the appropriate cells (reviewed in Kennison, 1993), and genes of the *Polycomb*-group (*Pc*-G), which keep homeotic selector genes repressed in those cells in which they were initially not activated (reviewed in Bienz, 1992; Paro, 1993). *Pc*-G loss-of-function mutants show typical homeotic transformations, reminiscent of gain-of-function mutations of homeotic genes. These transformations have been shown to result from the ectopic expression of homeotic selector genes outside of their initial domain of expression (Struhl and Akam, 1985; White and Wilcox, 1985; Simon et al., 1992). Genetic analysis suggested that there are 30 to 40 members of the *Pc*-G (Jurgens, 1985). Seven of them have been molecularly characterized (reviewed in Orlando and Paro, 1995; Simon, 1995): *Polycomb* (*Pc*) (Paro and Hogness, 1991), *Posterior sex combs* (*Psc*) (Brunk et al., 1991; Van Lohuizen et al., 1991), *polyhomeotic* (*ph*) (DeCamillis et al., 1992), *Enhancer of zeste* (*E(z)*) (Jones and Gelbart, 1993), *Polycomb-like* (*Pcl*) (Loonie et al., 1994), *extra sex combs* (*esc*) (Gutjahr et al., 1995; Sathe and Harte, 1995) and *Sex comb on midleg* (*Scm*) (Borneman et al., 1996).

Based on sequence similarities between the *Pc* gene product and the *Drosophila* HP1 protein encoded by the *Su(var)205* gene (Eissenberg et al., 1990; Paro and Hogness, 1991), it has been proposed that *Pc*-G genes might function analogously to modifiers of Position-Effect Variegation (PEV) in the establishment or maintenance of chromatin structure (for reviews, see Orlando and Paro, 1995; Pirrotta, 1995). Pc-G proteins are thought to form multiprotein silencing complexes, repressing target genes by modifying higher-order chromatin structure. Supporting this model, several *Pc*-G gene products, including PC, PH, PCL and PSC, show partial or complete overlapping

binding patterns on polytene chromosomes (DeCamillis et al., 1992; Franke et al., 1992; Paro and Zink, 1992; Martin and Adler, 1993; Loonie et al., 1994). Furthermore, immunoprecipitation experiments showed that PC and PH proteins are associated in large multiprotein complexes (Franke et al., 1992). Despite similarities between the *Pc*-G mutant phenotypes, the known members of the *Pc*-G show distinct molecular and functional properties. PC shares with HP1 a short conserved region, the chromodomain, required both for PC functioning as a repressor and PC binding to chromatin (Messmer et al., 1992). PH and SCM proteins contain zinc-fingers (DeCamillis et al., 1992; Bornemann et al., 1996). ESC is a member of the WD-repeat family (Gutjahr et al., 1995; Sathe and Harte, 1995; Simon et al., 1995) and, as such, might be involved in mediating protein-protein interactions. Because of this structural and possible functional heterogeneity, further understanding of the molecular basis of *Pc*-G-mediated silencing will require the molecular characterization of other members of this group of genes.

In this paper, we describe the cloning and characterization of the *cramped* (*crm*) gene of *Drosophila*. Based on several characteristics, *crm* can be classified as a *Pc*-G gene. Analysing the cellular localization of the CRM protein revealed striking similarities with the distribution of the Proliferating Cell Nuclear Antigen (PCNA), a protein involved in DNA replication. Furthermore, we show that *crm* and *mus209*, the *Drosophila* gene encoding PCNA, interact genetically, highlighting possible interactions between *Pc*-G-mediated silencing and DNA replication.

MATERIALS AND METHODS

Fly stocks

The *crm* gene has received several names, like *stubarista*, *sparse arista* and *lethal (1) zeste-white 9*, before the nomenclature was settled by Lindsley and Zimm (1992). Fly stocks used were crm^{7} (Shannon et al., 1972a,b), crm^{sa} (Rayle and Green, 1968), Pc^{3} (Haynie, 1983), $mus209^{B1}$ (Henderson et al., 1994). Df(1)X12 is described in Judd et al. (1972), Df(1)X22 was kindly provided by B. Judd. Recipient stocks for germ-line transformation were $y\text{-}ac\text{-}w^{1118}$ and ry^{506}. All flies were raised on standard medium supplemented with baker's yeast, at 25°C unless specified in the text. Oregon-R was used as wild-type strain. The effect of crm^{sa} mutation on *white* variegation in w^{m4h} flies was quantified by measuring the relative red eye pigment content according to Ashburner (1989).

Isolation of the *cramped* gene

Preparation of genomic DNA, isolation of DNA from λ phages and plasmids, restriction endonuclease digestion, gel electrophoresis of DNA and Southern-blotting, and DNA sequencing were done using standard protocols (Sambrook et al., 1989). Genomic clones w16, 28P2, 018A and 6A3 were kindly provided by M. Goldberg, clones M505, M512, M510 and M365 are described in Goldberg et al. (1982). Clone c10-22 was isolated from a genomic *Drosophila* Oregon-R library prepared in the λDASH II vector (provided by C. Wilson). The cDNA clones were isolated from a 3-12 hours embryonic cDNA library (a gift of L. Kauvar). Genomic and cDNA fragments were cloned into Bluescript vectors (Stratagene).

Plasmid and transposon constructs

To produce a truncated form of CRM protein (lacking 348 amino acids at the N terminus and 25 at the C terminus) in *E. coli*, a *Bam*HI-*Xho*I fragment was inserted into the pRSET-B expression vector (Invitrogen), in frame with a 6×His-tag. A truncated CRM protein, lacking 50 amino acids at the N terminus and containing an N-terminal 6×His-tag, was produced using a baculovirus system as follows. First, a *Sac*I-*Hin*dIII fragment was inserted into pRSET-A (Invitrogen), then cut with *Nde*I-*Hin*dIII, filled in with DNA polymerase and inserted into pBacpAK1 vector (Clontech) previously cut with *Bam*HI and blunted with *E. coli* DNA polymerase. This construct was used to infect SF21 insect cells following manufacturer's recommendations (Clontech). Full-length *Antennapedia* (*Antp*) cDNA was first cloned into pRSET vector, then inserted into pBacpAK1.

Transposon constructs for P-element-mediated germ-line transformation were made by inserting either the full-length *crm* cDNA sequence into the pCaSpeR3 vector (Thummel and Pirrotta, 1991), or by inserting a 14 kb *Xba*I genomic DNA fragment into the transformation vector C20-1 (a gift of U. Walldorf). This latter construct was further digested with *Hpa*1 and religated. The resulting construct (termed *rescue (–)*, see Fig. 2B) lacks almost the entire *crm* coding sequence. P-element-mediated germ-line transformation was done using standard procedures (Spradling and Rubin, 1982a,b).

Antibodies and western blotting

Antibodies

Truncated CRM protein was produced in the BL21 *E. coli* strain essentially as described elsewhere (Girard et al., 1991). Protein was purified following two different methods, either using nickel beads (Qiagen), or resolubilization from inclusion bodies. Purified proteins were injected into rabbits and antisera were affinity-purified using truncated CRM protein immobilized on CNBr-activated sepharose beads (Pharmacia). Monoclonal anti-PCNA was purchased from Boehringer. Polyclonal rabbit anti-*Drosophila* PCNA antiserum was a gift of P. Fisher (Ng et al., 1990). Rat anti-HAIRLESS (H) was a gift of A. Preiss.

Western blotting

Cell extracts were prepared from bacteria or baculovirus infected cells carrying *crm* or full-length *Antp* constructs, by taking up cells into lysis buffer (Laemmli, 1970). Third instar larvae carrying the hs-*crm* transgene were heat-shocked at 37°C for 1 hour, allowed to recover for 3 hours at 25°C, then heat-shocked again for 1 hour. Larvae were homogenized in lysis buffer, boiled and centrifuged for 10 minutes at 10,000 revs per minute. Wild-type larvae were prepared under the same conditions, omitting the heat-shock procedure. All samples were run on 8% SDS-PAGE. Volumes equivalent to three heat-shocked larvae and ten non-heat-shocked were loaded on the gel. Separated proteins were transferred to nitrocellulose (Schleicher & Schuell). Affinity-purified rabbit anti-CRM was used at a dilution of 1:500, in PBS containing 1% bovine serum albumin and 0.05% Tween 20, and detected using the ECL kit (Amersham) and HRP-conjugated swine anti-rabbit antibodies (Dakopatts) diluted 1:2,000.

Embryo and tissue immunostaining

After dechorionation, embryos were fixed in formaldehyde and prepared for immunostaining using standard procedures (Ashburner, 1989). For Fig. 4, affinity purified anti-CRM was used at a dilution of 1:500 and detected with HRP-conjugated swine anti-rabbit (Dakopatts). For Fig. 5, rabbit anti-CRM and rat anti-H, both diluted 1:100, were used for double immunostaining. Secondary antibodies used were FITC-conjugated anti-rabbit (Dakopatts) and rhodamine-conjugated anti-rat (Cappel). DNA was stained with DAPI. For Figs 4 and 7, antibody staining of salivary glands from third instar larvae was done as described (Bone et al., 1994), with a fixation time of 20 minutes. For Fig. 7, fixed glands were double stained with rabbit anti-CRM mixed with monoclonal anti-PCNA. Secondary antibodies used were rhodamine-conjugated anti-rabbit (Dakopatts) and FITC-conjugated anti-mouse (Pierce). Immunofluorescent stainings were observed by confocal microscopy.

RESULTS

cramped is a *Polycomb*-group gene

The phenotype of *crm* mutants was initially described by Rayle and Green (1968). Typically, hemizygous mutant males or homozygous females die late in development, with lethality periods ranging from third instar to late pupal stages. Homozygous female escapers are sterile and have rudimentary ovaries with no oocytes beyond stage 7 (Shannon et al., 1972a,b). Mutants show a typical altered morphology of the antennae: swollen antennae, short aristae with fewer branches than in wild type. Male pharate adults have extra sex comb teeth on the second tarsal segment of prothoracic legs (Fig. 1D), on the first tarsal segment of mesothoracic legs (Fig. 1E), and occasionally on the first tarsal segment of metathoracic legs (Fig. 1F). Very rarely, a single ectopic sex comb tooth is found on the second tarsal segment of mesothoracic legs. The apparition of ectopic sex comb teeth on second and third legs represent partial homeotic transformations, similar to those observed in mutants of the *Pc*-G. In addition to these *Pc*-like transformations, *crm* mutants also exhibits partial distal-to-proximal transformations, mostly visible on the first leg (i.e. ectopic sex comb teeth on the second tarsal segment). An intrinsic characteristic of the *Pc*-G members is that mutations in these genes interact synergistically, double mutant combinations resulting in more severe phenotypes than those that would be expected if their effects were additive (Jurgens, 1985). This also appears to be the case for *crm*. *Trans*-heterozygous males of crm^7 with Pc^3 die as pharate adults, with second and third legs completely transformed towards the first. This is easily scored with the apparition of complete sex combs on the mesothoracic (Fig. 1H) and metathoracic legs (Fig. 1I), and the presence of ectopic transverse rows at the end of the tibia of second and third legs (not shown). Additionally, ectopic sex comb teeth are also found on the second tarsal segment of all legs (Fig. 1G-I). These phenotypical observations led us to classify *crm* as a member of the *Pc*-G. In support of this, we have observed that the homeotic gene *Sex comb reduced* (*Scr*) was expressed outside of its normal domain of expression in crm^7 third instar larvae (data not shown), as it was already shown for *Pc* (Pattatucci and Kaufman, 1991).

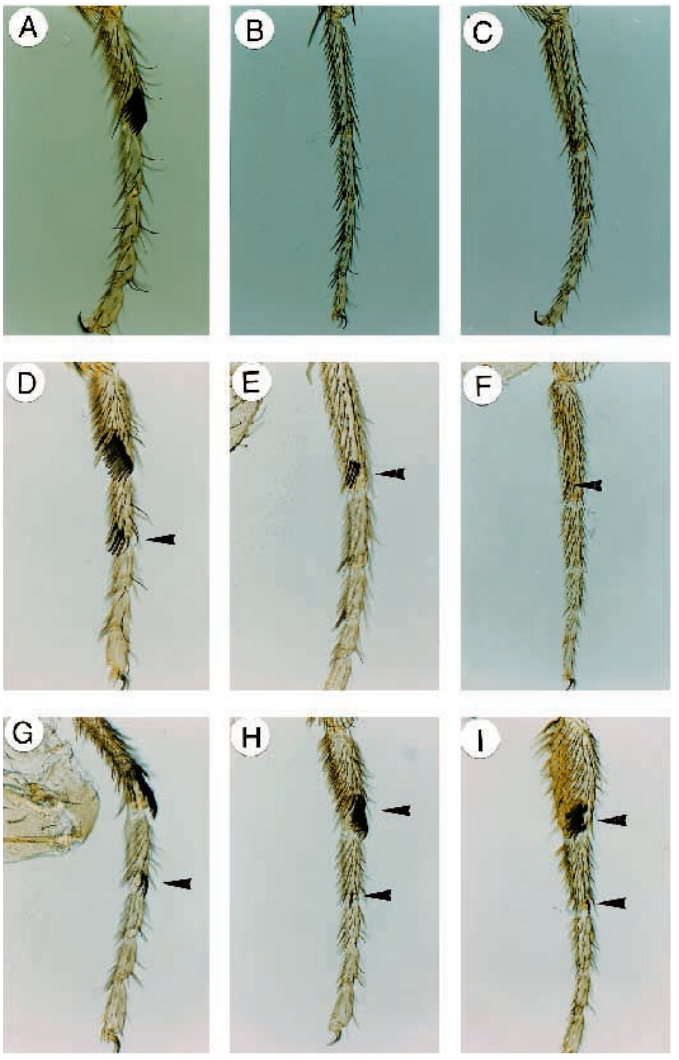
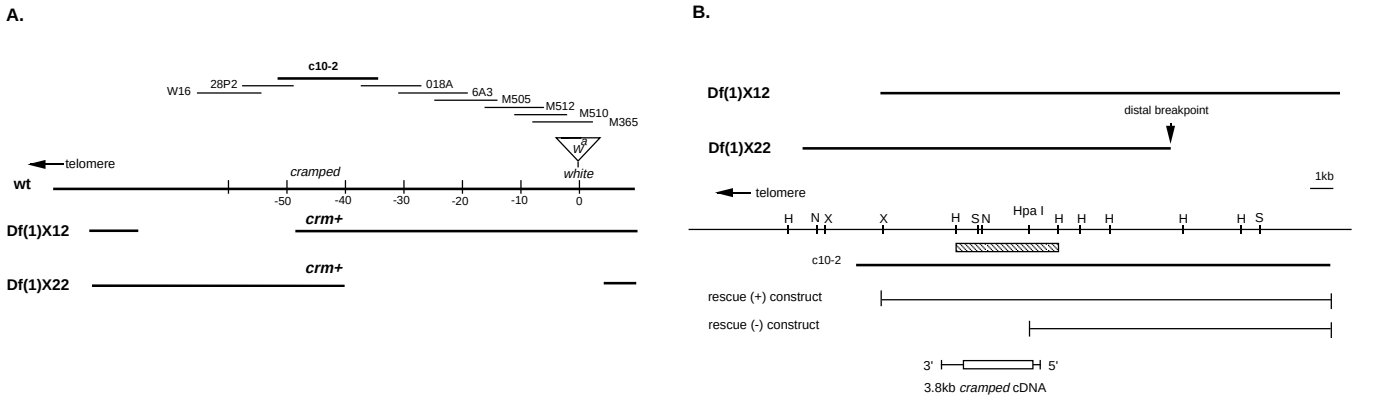


Fig. 1. *cramped* genetically interacts with *Polycomb*. Photomicrographs of the distal portion of prothoracic (A,D,G), mesothoracic (B,E,H) and metathoracic legs (C,F,I) from wild-type (A-C), hemizygous crm^7 (D-F) and crm^7/Y;Pc^3/+ (G-I) male pharate adults. Arrowheads point to additional sex comb teeth.

Isolation of the *cramped* gene

crm has been mapped to the cytological position 3C1, adjacent to the *white* locus (Rayle and Green, 1968; Judd et al., 1972; Shannon et al., 1972a,b). For the precise localization and the subsequent cloning of the *crm* gene, we have used two deficiencies, Df(1)X12 and Df(1)X22 (Judd et al., 1972), which both complement *crm* mutants. The distal breakpoint of Df(1)X22 has been mapped within a region –50 to –45 kb from the w^a insertion site (see Fig. 2A). The proximal breakpoint has not been mapped physically, but Df(1)X22 uncovers the *white* locus (B. H. Judd, personal communication). Although the proximal breakpoint of Df(1)X12 is not mapped, it has been reported that Df(1)X12 uncovers all lethal *zw* complementation groups, except *crm* (zw^9), which is most proximally located (Thierry-Mieg, 1982). It thus appeared that the *crm* gene was located between the proximal breakpoint of Df(1)X12 and the distal breakpoint of Df(1)X22. To clone the genomic DNA covering the region between these two breakpoints, we used a chromosome walk strategy, starting from the distal end of the genomic clone M505 (see Fig. 2A) previously isolated in the laboratory (Goldberg et al., 1982). Clone c10-2 was found to reach the proximal end of another contig of clones covering the *zw* region (Goldberg et al., 1982). In order to identify the *crm* gene, rescue experiments were performed. An 14 kb *Xba*I fragment from clone c10-2 (see Fig. 2B) was found to rescue all *crm* mutant phenotypes (including antennal and leg defects, and female sterility) when introduced by germ-line transformation. As a control, almost the entire *crm* coding region was removed from the initial rescue construct (see Fig. 2B). We found that this construct did not rescue any of the *crm* phenotype.

To identify transcription units inside the 14 kb genomic fragment, whole-mount in situ hybridisations were performed with five *Hin*dIII restriction fragments. A strong signal was obtained only with the 3.6 kb fragment (see Fig. 2B). This fragment was used to screen a 0-8 hours embryonic cDNA library, from which we isolated a full-length *crm* cDNA clone.



C.

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-76                                    ggaagactacgcagtttaattgatattgccattgattgatcctgtctaggaggcaagcggatgtggattggccaac
1    atggaggaactgtccaagcagccgccgccgccaccccgctgacgcagccgccgccgccgtcgtcatccgtgtccattgaggagccgctgccaaatggcaagggagga
1     M  E  E  L  S  K  Q  P  P  P  P  P  L  T  Q  P  P  P  P  S  S  S  V  S  I  E  E  P  L  P  N  G  K  G  G

106  ggagcagtagttgttaactcgatagctaagctgcccgaggaggagctcctcggctcggtgaccatgcacaattgtccgggaacgcgggccagtgcccgggtcata
36    G  A  V  V  V  N  S  I  A  K  L  P  E  E  E  L  L  G  S  V  T  M  H  N  C  P  G  T  R  A  S  A  R  V  I

211  cagaagatgaagcaggaccagacgcgcccgatgacaccgccgcccagcgaacgggagccgaacaaaaaggaggagaaggccgcccaaaagacgcccagccagctg
71    Q  K  M  K  Q  D  Q  T  R  ***P  M  T  P***  P  P  S  E  R  E  P  N  K  K  E  E  K  A  A  Q  K  T  P  S  Q  L

316  aagacgggcagcgggaagaccacctggacgaatgtggagcgcaactgcttcttcgacgccctcaacgaattcggcaaggacttcgaggcggtggccaactgcatc
106   K  T  G  S  G  K  T  T  W  T  N  V  E  R  N  C  F  F  D  A  L  N  E  F  G  K  D  F  E  A  V  A  N  C  I

421  aatgccaagctgaagcgccgcaatgccaacagcgactatagtttcaaaaccaaggaccaagttcgccagcactattaccagacccatcacaagatttgcaaatat
141   N  A  K  L  K  R  R  N  A  N  S  D  Y  S  F  K  T  K  D  Q  V  R  Q  H  Y  Y  Q  T  H  H  K  I  C  K  Y

26  gtgcgcttctcggaggaactgaaaaagcccgcccaggagttgtacacgctgatcaactacggcgagatgcggcgcaagctgcagttcctcacggagaagcacttc
176   V  R  F  S  E  E  L  K  K  P  A  Q  E  L  Y  T  L  I  N  Y  G  E  M  R  R  K  L  Q  F  L  T  E  K  H  F

631  atgaagctgaagcagctggtctaccagggccaaataaccgtgcgttgcaagggcaagaacattcgcattaagacgccatcgtgcaaggcgctccggcggctaaat
211   M  K  L  K  Q  L  V  Y  Q  G  Q  I  T  V  R  C  K  G  K  N  I  R  I  K  T  P  S  C  K  A  L  R  R  L  N

736  caattggatgactcactggaggatatccggctgcccagcaaggtggaggtgctggtgactcccgccaatatggaggccttcggacgcgtccagtcactggcgcag
246   Q  L  D  D  S  L  E  D  I  R  L  P  S  K  V  E  V  L  V  T  P  A  N  M  E  A  F  G  R  V  Q  S  L  A  Q

841  aatccacgcggcaggatcatagttccgctgcacaagaaactaatcagcttcatcaagacgttcgagtacaaatggcgcagcgccaatcagcgtttgcacgaggag
281   N  P  R  G  R  I  I  V  P  L  H  K  K  L  I  S  F  I  K  T  F  E  Y  K  W  R  S  A  N  Q  R  L  H  E  E

946  aagagtgcatacttttccagcagcctgccatctgctgcgtccaacaacaataacaacaacaatgaaacggaaccattacagccgtcagttgcatcgctggatccc
316   K  S  A  Y  F  S  S  S  L  P  S  A  A  S  N  N  N  N  N  N  N  E  T  E  P  L  Q  P  S  V  A  S  L  D  P

1051 tcaatgtgcttccagccccggccgggagtggccattcaccgacctttgctcagcatcaccgcctacctaagcagcatcagtatttgcttgacggcatacgaggag
351   S  M  C  F  Q  P  R  P  G  V  A  I  H  R  P  L  L  S  I  T  A  Y  L  S  S  I  S  I  C  L  T  A  Y  E  E

1156 cgcatgggcttcaaagtgcgcagcgagaccttgggcaatttggccggcatgtcggtggcggccaacaagcgactacgaacggaaagcggctccgagaagcgttcg
386   R  M  G  F  K  V  R  S  E  T  L  G  N  L  A  G  M  S  V  A  A  N  K  R  L  R  T  E  S  G  S  E  K  R  S

1261 ccggagacaaagaaacccaaatcgagtgccagcccgccgctggagaagagtttggacgacgggcccttggagggcaatctgatgaagatggagaatagcagcggt
421   P  E  T  K  K  P  K  S  S  A  S  P  P  L  E  K  S  L  D  D  G  P  L  E  G  N  L  M  K  M  E  N  S  S  G

1366 gatgagctgggcgaggagattcatgagtttctgggcgatatattggaggccatgcagcatccgcaagcagccactattccagctctttcggccaccactggtgat
456   D  E  L  G  E  E  I  H  E  F  L  G  D  I  L  E  A  M  Q  H  P  Q  A  A  T  I  P  A  L  S  A  T  T  G  D

1471 accacaactgttgctgttgcgcttgaaacatcgcacgatcctgtacaacaggcttatccggccaacgcggatctatctaatgccatggcgacatctgttctacaa
491   T  T  T  V  A  V  A  L  E  T  S  H  D  P  V  Q  Q  A  Y  P  A  N  A  D  L  S  N  A  M  A  T  S  V  L  Q

1576 acttcctgtgctgcagctcccgctccttcgactcctgtaaccggttctttggcggctccatccgtagcccgctccaaacgcaaagaagctaaggaggcggcggcc
526   T  S  C  A  A  A  P  A  ***P  S  T  P***  V  T  G  S  L  A  A  P  S  V  A  R  S  K  R  K  E  A  K  E  A  A  A

1681 gcggcacaggctcgaaatttaagccactgctcagcgacgatatactgaagagaatccgcaagggctggacgcaggcgaatgcggcggatataaccatcggtgat
561   A  A  Q  A  R  N  F  K  P  L  L  S  D  D  I  L  K  R  I  R  K  G  W  T  Q  A  N  A  A  D  I  T  I  G  D

1786 ctgtatgtcgtttttggccaggattccaagctggagctagagtattattggtgtgaggtcgacagttcgacggccatggcatccagcatactaacaatcaacacg
596   L  Y  V  V  F  G  Q  D  S  K  L  E  L  E  Y  Y  W  C  E  V  D  S  S  T  A  M  A  S  S  I  L  T  I  N  T

1891 gtaacgcccagctcctcgtcggtgggcacccaaaccggcagttccgcctcgaacgccaaccaaacaagcgcatcttccaactgctacgtcagcgcatcgagcaat
631   V  T  P  S  S  S  S  V  G  T  Q  T  G  S  S  A  S  N  A  N  Q  T  S  A  S  S  N  C  Y  V  S  A  S  S  N

1996 tcgagcacctcgagcacttcgctgccctacatcccaacgactgtgatagcgtggaacgcgtcagggccgtcaccacatcgtcggtgagcaacaagctgaagcacc
666   S  S  T  S  S  T  S  L  P  Y  I  P  T  T  V  I  A  W  N  A  S  G  P  ***S  P  H  R*** R  *

2101 tgctactcgtggccaatctcagcgagcgcgtgcgcaagcgccaatgcaactgcggccacacgtgcgatcgcaagcgggacctgatgaccaaggcgcagcagctgg
2206 cagaggcgacggccaccggcggagtgggcggcatggttgagggcaatttccgaacgccgatgctgccagtgcggcggccgatatccaacatcgatcctgtgcgcc
2311 aactctcagcgctcacacggcaaaagatcagtcgtcaggtgcttgtgcagcgccgtctcctaccacccacatccgctggcgatcgtccgtacgatctgctgagtg
2416 tgcgccaactccacagtggccttttcgaaccgattgatcgcgtggatggcacaaagttccgctggcatcagcacctctggctccgaaacccgattgcagcatgaat
2521 gccatgaccgcttcacaggatcaagagccgggggatcagggagcattggagttcctcaacgacgaggccacgcaggtcagcgctcgagacatgcccaatctggac
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2731 cacttgctcagagattcaacatccaacgcacgctggctggaggacaatatcaatgacttctcgttgacgtcgctgctggggcatttggatgagattgatgccacc
2836 agggatatactggatccgtcgtcgagcatgtcggtgatcagcgagagcagcgtggaccttcggcacaagttccaggaaatcgccgccctgctgcagcagcaggag
2941 aaagattagcagtggccactgcggcaatccggtcgtgtcctcttcgtttgtgtacccaagtccggatcttgagatgagagtcgtccaagctggacgatggaaagg
3046 aaagccacgtgtgcctccaagcctcatgtcccactgtattactcattactcactcatcccacccacccacgttatacccactaacatttatgtttttacgcgact
3151 ttttgtgatctatactcatttttatgtatgccaagtattagcaagcactcacattgtctgaacatatatatttatttatacattaactttatgtatcgcgcggca
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3361 gttcaatgcagcatagtagcttagcttaaggtccccacacccgcacccgcaccctcaccctagccactatcaactacaaaacccaatcgaaacttccatatgta
3466 tagagattctcccgcctccacctgataaactaaagctcattacattaaatcaaatcgaaaggatcgtcagaagactcgaaatgtttgttgaccatatataatata
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3676 aa***aataaa***agcgaattgaactaaaactaaaaaaaaaaaaaaa
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Fig. 2. Isolation of the *cramped* gene. (A) Chromosome walk. The distance is measured in kb starting from the w^a (*white apricot*) insertion site. The positive direction is towards the centromere, the negative towards the telomere. Genomic clones used are aligned above the map. Below is a schematic representation of the two deficiencies used in this study. (B) Molecular map of the *cramped* locus. The 15 kb insert of clone c10-2 is indicated by a solid line below the map. The 3.6 kb *Hin*dIII fragment used for the screening of the cDNA library is indicated by a hatched box. The genomic fragments used for rescue experiments are shown below the map. The *crm* cDNA is shown at the bottom, with the open reading frame represented as an open box, and 5′ and 3′ untranslated regions as solid line. Restriction sites: H, *Hin*dIII; N, *Not*I; S, *Sal*I; X, *Xba*I. (C) Nucleotide sequence of *crm* cDNA and predicted amino acid sequence of the *crm* gene product. Underlined are three potential nuclear localization signals (residues 143-147, 549-553 and 577-581) and a putative polyadenylation signal. Bold italic letters represent consensus phosphorylation sites for MAP kinase (P-X-S/T-P) and cyclin-dependent kinases (S/T-P-X-basic). Five consensus sites for phosphorylation by casein kinase II (S-X-X-E) are present at positions 24, 86, 414, 454 and 604. The accession number of the sequence reported here is Y13674.

Transcripts corresponding to this cDNA were further shown to exhibit embryonic expression pattern identical to the 3.6 kb genomic fragment (data not shown).

The *crm* cDNA sequence

The nucleotide sequence of *crm* full-length cDNA clone is shown in Fig. 2C. The sequence immediately preceding the initiation codon (CAAC) is similar to the *Drosophila* consensus sequence for translation start sites (C/AAAC/A) (Cavener, 1987). The *crm* open reading frame predicts a protein of 693 amino acids, with a calculated relative molecular mass (M_r) of 75×10^3. Searching the available databases, we have found no significant similarities to known proteins. Nevertheless, the CRM protein sequence shows interesting features. PEST sequences, which are thought to be involved in protein degradation, are present in the N-terminal region (residues 2-56) and in a central region of the CRM protein (residues 412-437). Three putative nuclear localisation signals are present (underlined in Fig. 2C). The CRM protein sequence contains several consensus sites for phosphorylation (Kennelly and Krebs, 1991), by MAP kinase (P-X-S/T-P), cyclin-dependent kinases (S/T-P-X-basic) (bold in Fig. 2C) and casein kinase II (S-X-X-E). The C-terminal region is rich in serine and threonine residues (45% over the last 77 amino acids). Finally, CRM contains a region with high density of alanine residues (starting at position 529), with a stretch of 36 residues containing 14 alanine (40%). Alanine-rich regions are present in the *engrailed* and *Kruppel* gene products, and are involved in the transcriptional repressing activity of these proteins (Han and Manley, 1993). Furthermore, another *Pc*-G gene product, *Scm*, was shown to contain similar alanine-rich sequences (Bornemann et al., 1996).

Developmental expression of the CRM protein

In order to examine the expression pattern of the *crm* gene product, we have raised polyclonal antibodies in rabbit against a truncated CRM protein made in *E. coli*. These antibodies were affinity-purified and their specificity was tested by western blotting. As shown in Fig. 3, anti-CRM detect truncated CRM proteins expressed in bacteria (lane 1) and baculovirus-infected insect cells (lane 3). The protein made using the baculovirus system, which is deleted of the 50 N-terminal amino acids and contains an in-frame 6×His tag, runs on SDS-PAGE with an apparent M_r of 95×10^3, which is higher than the calculated M_r of 75×10^3 for the full-length CRM protein. Endogenous CRM protein is detected in extracts from wild-type third instar larvae (lane 5) with an apparent M_r around 110×10^3. These differences between calculated and apparent M_rs might reflect post-translational modifications, since the CRM sequence contains several consensus sites for phosphorylation (Fig. 2C). Additionally, full-length CRM protein induced in larvae under the control of a heat-shock promoter migrates exactly as the 110×10^3 M_r band (data not shown). Controls with bacteria transfected with empty vector (lane 2) or baculovirus-infected cells expressing ANTP protein (lane 4) show no cross-reactivity, confirming the specificity of the anti-CRM antibodies.

We used these affinity purified anti-CRM antibodies to examine the developmental expression pattern and subcellular localization of the CRM protein. Strong expression was detected in the nuclei during early embryogenesis (Fig. 4). During nuclear multiplication, CRM was found present in nuclei throughout the embryo (Fig. 4A). Nuclear staining persists at a similar level during cellularization (Fig. 4B) and at the time of gastrulation (Fig. 4C). We also observed significant staining in the pole cells (this staining is not visible in Fig. 4B, because of different focal planes). Later in development, a strong staining is visible in the central nervous system (CNS) and the gonads (not shown). As a control, CRM antibodies preincubated with purified CRM protein did not reveal any nuclear staining. Whole-mount in situ hybridisation of embryos confirmed these observations and, additionally, revealed a strong maternal accumulation of *crm* transcripts (data not shown). Finally, we have observed a strong immunostaining in polytene nuclei of salivary glands of third instar larvae (Fig. 4D).

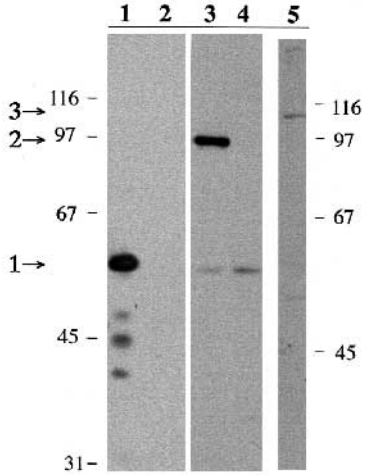


Fig. 3. Specificity of the anti-CRM antibody. The specificity of the affinity-purified anti-CRM antibody was tested by western blot analysis. A positive signal is detected in extracts from bacteria expressing the truncated CRM protein used for immunization (lane 1), in baculovirus-infected insect cells expressing a truncated CRM protein lacking 50 amino acids at the N terminus (lane 3) and in total extract from wild-type third instar larvae (lane 5). Control lanes show absence of immunoreactivity in bacteria transfected with the empty expression vector (lane 2) and in baculovirus-infected cells expressing full-length ANTP protein (lane 4). Arrows point to truncated CRM proteins (1 and 2) and to endogenous full-length CRM (3). Relative molecular masses are shown ($\times10^{-3}$).

Cell-cycle-dependent localization of the CRM protein

In our initial embryo immunostaining experiments with anti-CRM antibodies, we observed that a significant proportion of early embryos showed little or no staining and that, at the time of gastrulation, strong staining was clearly distributed in a region-specific manner. We thus questioned whether this dynamic pattern of CRM staining might reflect cell-cycle-dependent changes in CRM protein localisation. For this purpose, we performed immunostaining of embryos at mitotic cycle 8 with anti-CRM, DAPI and anti-H antibody. During the first 13 zygotic division cycles, most of the embryo is syncytial, with the exception of the pole cells that form in cycle 10. During these preblastoderm cycles, nuclei divide nearly synchronously and the cell cycle consists of alternating S- and M-phases without G1- and G2-gap phases (reviewed in Foe et al., 1993). As shown in Fig. 5A, all interphase nuclei are strongly stained with anti-CRM antibodies. At the beginning of chromosome condensation, but before nuclear envelop breakdown, CRM immunostaining is still associated with the nucleus (Fig. 5D). In prophase, after nuclear envelop breakdown occurred, CRM immunostaining completely disappeared (Fig. 5G). CRM protein was clearly not associated with chromosomes during metaphase (Fig. 5J) and anaphase (Fig. 5M). CRM immunostaining reappeared at telophase, concomitant with the formation of the two daughter nuclei (Fig. 5P). In marked contrast, H immunostaining appeared to be present uniformly in all somatic nuclei and clearly remains associated with the condensed chromosomes during all stages of mitosis (Fig. 5C,F,I,L,O,R). At the time of gastrulation, mitosis is no longer synchronous, with groups of cells, the so-called mitotic domains, entering mitosis at different times. This results in precise and reproducible patterned mitoses replacing the prior global mitotic waves (Foe, 1989). It was possible to see cell-cycle-dependent nuclear localization of CRM protein in single embryos at this stage, with cells in interphase staining strongly and cells in metaphase showing no CRM immunostaining (data not shown). We conclude from these experiments that CRM protein localizes to the nucleus mainly during S-phase in early embryogenesis and is not associated with chromatin during mitosis.

Co-localization of CRM and PCNA proteins

The S-phase-specific nuclear localization of CRM is reminiscent of that of the PCNA (Proliferating Cell Nuclear Antigen) protein in *Drosophila* embryos. PCNA is a highly conserved protein essential for DNA replication and repair (reviewed in Fairman, 1990). During the first 13 nuclear division cycles, PCNA is present in all interphase nuclei and absent from metaphase chromosomes (Yamaguchi et al., 1991). Double immunostaining for CRM and PCNA revealed that the timing of appearance and disappearance of the nuclear signal are identical during the preblastoderm cycles and that, at the time of gastrulation, both proteins shows overlapping patterns of expression (data not shown). The co-localization of these two proteins was clearly observed in polytene tissues. Fig. 6 shows immunostaining for CRM (Fig. 6A) and PCNA (Fig. 6B) in salivary gland nuclei from third instar larvae, which clearly overlap (Fig. 6C). By contrast, immunostaining with anti-PC antibodies revealed a banded pattern inside the nucleus, clearly different from the more diffuse chromosomal CRM/PCNA stainings (data not shown; Messmer et al., 1992). We conclude from these immunostaining experiments in embryos and larvae that CRM and PCNA proteins display overlapping expression patterns during embryogenesis, and appear to co-localize in polytene nuclei.

crm interacts genetically with *mus209* (PCNA)

In *Drosophila*, the *mus209* gene has recently been shown to encode the homolog of the mammalian PCNA (Henderson et al., 1994). *mus209*^{B1} mutants exhibit a complex pleiotropy of temperature-sensitive lethality, hypersensitivity to DNA-damaging agents and suppression of position-effect variegation (Henderson et al., 1994). By carefully examining pharate adults, we found that *mus209* mutant males showed a *crm*-like phenotype, with an ectopic sex comb tooth being present in the second tarsal segment of prothoracic legs, of approximately one third of homozygous *mus209*^{B1} males (Fig. 7A). We then examined leg phenotypes in *trans*-heterozygous *crm*^{7};*mus209*^{B1} flies. As illustrated in Fig. 7, we observed a strong enhancement of the *crm* phenotype. Indeed, complete sex combs are present on the first tarsal segment of all legs. Prothoracic legs show a strong distal-to-proximal transformation, with ectopic sex comb teeth formed on the second and third tarsal segments (Fig. 7B). Additional sex comb teeth are

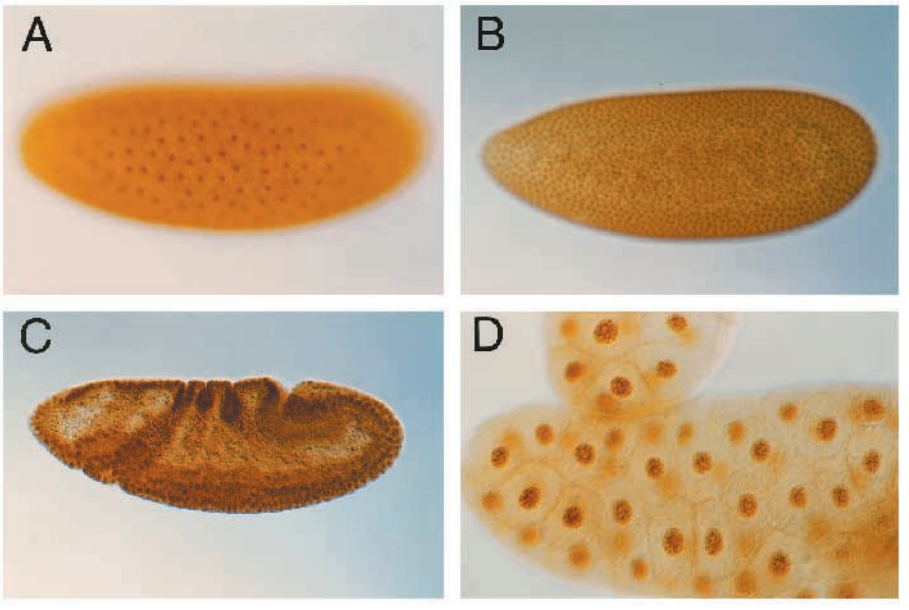


Fig. 4. Developmental expression of the *crm* gene product. Immunostaining of wild-type embryos and salivary glands from third instar larvae using affinity-purified anti-CRM antibody. The CRM protein is ubiquitously expressed throughout development and is localized to the nuclei. Shown are embryos at stage 2 (nuclear multiplication) (A), stage 4 (cellularized blastoderm) (B), and stage 8 (germ band extension) (C), and CRM nuclear staining in salivary glands from a wild-type third instar larva (D) (high magnification). Embryos are shown anterior to the left, dorsal to the top.

also observed on the second tarsal segment of mesothoracic legs (Fig. 7C). These morphological changes indicate that in a *crm*;*mus209* mutant background, both posterior-to-anterior and distal-to-proximal transformations are strongly enhanced.

Suppression of Position Effect Variegation in *crm^{sa}* mutant

Position Effect Variegation (PEV) refers to the variation of expression of a gene depending on its chromosomal position (for a recent review, see Karpen, 1994). The variegated phenotype associated with position-effect rearrangements, such as *white mottled 4* (w^{m4}), has been used successfully to screen for mutations in genes that encode structural or regulatory chromatin components. Some members of the *Pc*-G can influence PEV phenomena, and certain modifiers of PEV depict homeotic phenotypes, suggesting that *Pc*-G-mediated silencing and PEV might share several components (Grigliatti, 1991; Dorn et al., 1993). Additionally, *mus209* mutations were recently shown to suppress PEV (Henderson et al., 1994). For these reasons, we examined the effect of *crm* mutation on PEV associated with the w^{m4} chromosome inversion. As shown in Fig. 8, w^{m4} variegation leads to clones of phenotypically wild-type cells in a mutant background (Fig. 8A). This phenotype is suppressed in crm^{sa} *trans*-heterozygous (i.e. the amount of eye pigmentation is increased towards wild type) (Fig. 8B). Quantification of the red eye pigment levels revealed a suppressor ratio of 5.4, when comparing eye pigment levels in yw/w^{m4} and crm^{sa}/w^{m4} females. We conclude that *crm*, like *mus209* and certain members of the *Pc*-G, is a modifier of PEV.

DISCUSSION

cramped, a new member of the *Pc*-G

Based on genetic data, 40 loci have been identified as being part of the *Pc*-G, with only seven molecularly characterized to date. We report here the molecular isolation and characterization of the *crm* gene, a member of the *Pc*-G. *crm* mutants show homeotic transformations typical of the *Pc*-G, with second and third legs partially transformed towards the first. This phenotype is strongly enhanced in crm^{7};Pc^{3} mutants, suggesting synergistic interactions between

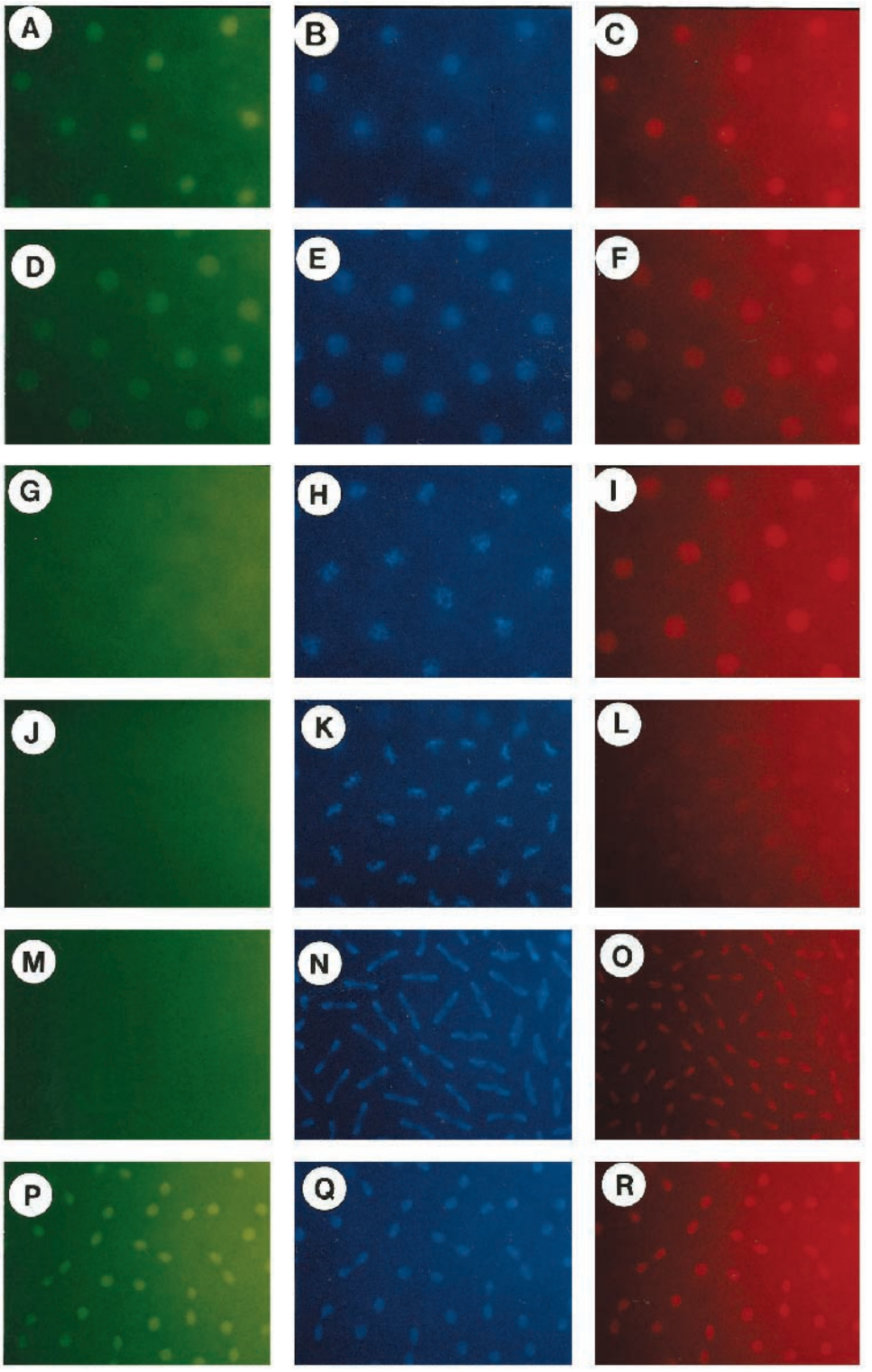


Fig. 5. CRM protein localization during the cell cycle. Triple staining of early wild-type embryos with rabbit anti-CRM (A,D,G,J,M,P), DAPI (B,E,H,K,N,Q) and rat anti-HAIRLESS (C,F,I,L,O,R). Shown are embryos stained at the period of rapid and synchronous cell cycles early in development, during interphase (A-C), early prophase (D-F), late prophase (G-I), metaphase (J-L), anaphase (M-O) and telophase (P-R).

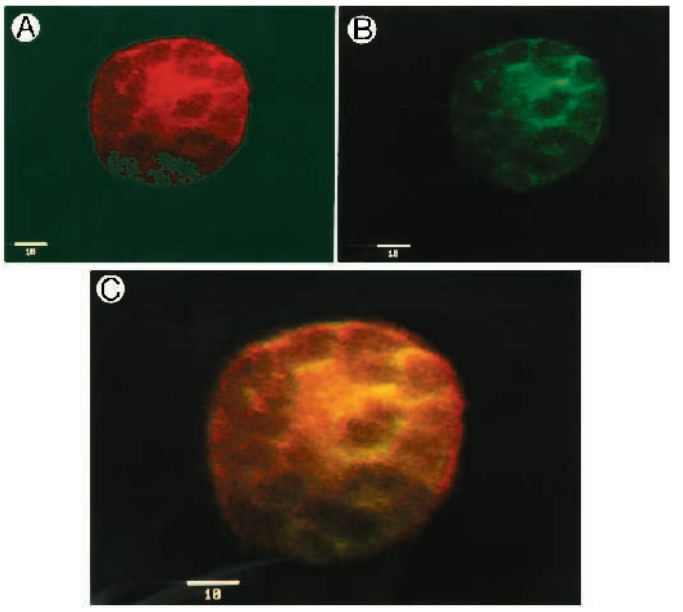


Fig. 6. Co-localization of CRM and PCNA in polytene nuclei of salivary glands. Shown are high magnification of a salivary gland polytene nucleus from a third instar larva, double-stained for CRM (A) and PCNA (B) with affinity purified rabbit anti-CRM and monoclonal anti-PCNA, respectively. These two images correspond to the same optical section. C, overlap of images shown in A and B. Bar represents 10 μm.

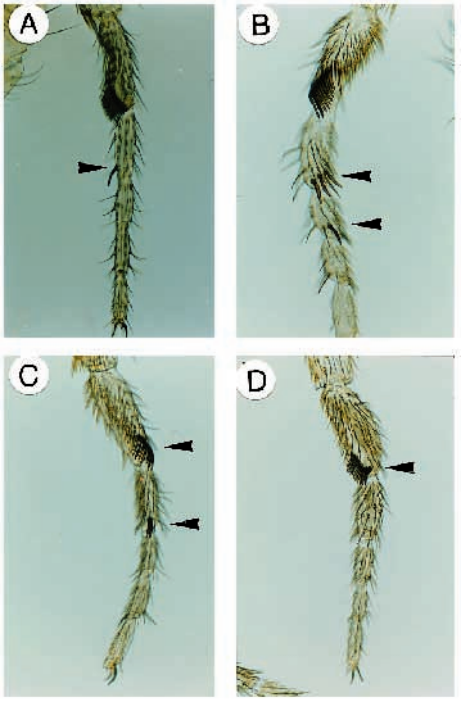


Fig. 7. *cramped* genetically interacts with *mus209* (PCNA). *mus209*^{B1} males were crossed to *crm*^{7}/FM7 females. The progeny was kept for 5 days at room temperature, then further incubated at 29°C. Photomicrographs of the distal portion of prothoracic (A,B), mesothoracic (C) and metathoracic legs (D) from +/Y; *mus209*^{B1}/*mus209*^{B1} (A) and *crm7*/Y; *mus209*^{B1}/+ (B-D) male pharate adults. Arrowheads point to additional sex comb teeth.

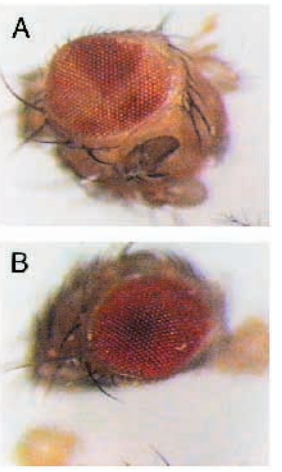
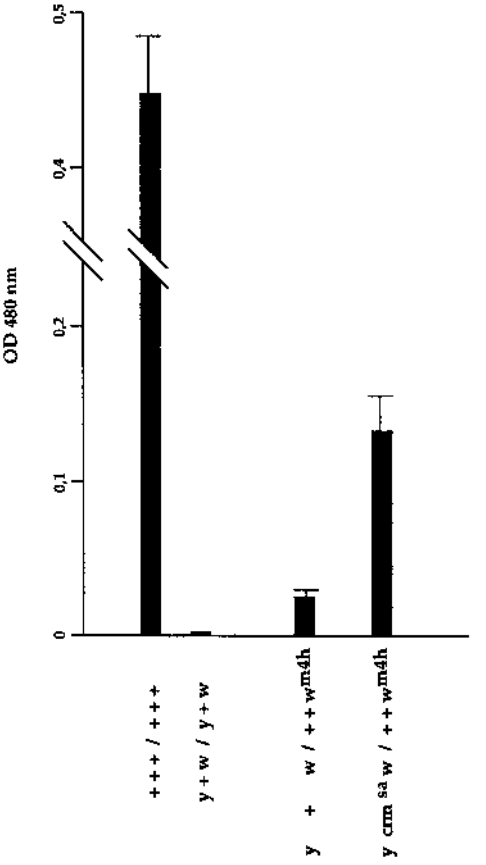


Fig. 8. Suppression of *white* variegation in *crm*^{sa} mutant. The effect of the *crm*^{sa} mutation on *white* variegation was assessed by crossing *crm*^{sa}/FM6 females to *w*^{m4h}/Y males. Females of the desired genotypes were collected over a 12 hours period and kept 5 days at 25°C. For quantification of the red eye pigment content, experiments were done in triplicate, on groups of 10 fly heads. Standard deviations are shown as thin lines over the bars. (A,B) Heads of representative flies of the following genotypes: (A)*y* + *w*/+ + *wm*^{4h} ; (B) *y crm*^{sa} *w*/+ + *w*^{m4h}.



these two genes. This phenotype might be explained, in part, by the derepression of the homeotic gene *Scr*, since ectopic *Scr* expression was observed in leg imaginal discs from *crm*^{7} mutant larvae. In addition to this common gain-of-function homeotic phenotype, mutants of the *Pc*-G show a wide range of individual phenotypes: segmentation defects, central nervous system defects and transformation along the dorsoventral axis. This suggests that the role of the *Pc*-G is rather general, preventing developmental regulators from being expressed in the wrong cells and tissues during development. Interestingly, *crm* mutants show transformation along the proximodistal axis (*i.e.* ectopic sex comb teeth on the second tarsal segment of prothoracic legs and, occasionally, of mesothoracic

legs). Although this phenotype is not a common characteristic of the *Pc*-G, it has been reported for some of its members, like *multi sex combs* (*mxc*) (Santamaria and Randsholt, 1995), *polycombeotic* (Jones and Gelbart, 1990) and *pleiohomeotic* (Girton and Jeon, 1994). All of these genes have yet to be molecularly characterized. As already suggested (Santamaria and Randsholt, 1995), common target genes, involved in controlling the proximodistal axis, might be affected by these mutations.

Viable homozygous *crm* mutant females are sterile, with oocytes arrested at early stages of differentiation. Together with the observation that *crm* expression is high in the gonads late in development, this suggests a possible involvement of *crm* in early gonad development. Other *Pc*-G proteins also appear to be required during oogenesis: *Pc* is strongly expressed during oogenesis (Paro and Zink, 1992) and *mxc* mutations were also shown to affect germ-line formation and differentiation (Santamaria and Randsholt, 1995).

Cellular distribution of the CRM protein

CRM protein is nuclearly localized throughout development. We have found that CRM nuclear staining is S-phase specific during early embryogenesis. CRM immunostaining is not visible from early prophase to anaphase and reappears at telophase in the two daughter cell nuclei, suggesting a possible degradation of CRM protein early in mitosis. This is further supported by the presence of several PEST sequences, known to be involved in protein degradation, in the CRM protein sequence. Based on our immunostaining data, we cannot exclude that a fraction of CRM protein is still present on condensed mitotic chromosomes, but not accessible to the anti-CRM antibodies. This particular cell-cycle-dependent nuclear localization is similar to that described for PCNA (Yamagushi et al., 1991). In contrast, PC (Messmer et al., 1992) and PSC (Martin and Adler, 1993) proteins appear to remain associated with condensed chromatin during mitosis. Furthermore, differences in CRM and PC protein localization were clearly visible in polytene nuclei from salivary glands, with anti-PC antibodies revealing a clear banded pattern and anti-CRM a more diffuse chromosomal staining. The products of the *Pc*-G genes are thought to form multiprotein complexes, repressing target gene expression by maintaining a closed chromatin configuration. According to this model, some *Pc*-G proteins, like PC, PH, PCL and PSC, have been shown to share, completely or partially, binding sites on polytene chromosomes. Immunostaining experiments revealed that CRM proteins bind around 50 discrete sites on chromosomes (Y. Y., unpublished observations). The precise determination of these CRM-binding sites is under investigation and should give clues to the identification of potential target genes.

cramped/PCNA interaction, a link between *Pc*-G-mediated silencing and DNA replication?

We have provided several lines of evidence for a possible *crm*/PCNA interaction. First, both genes show similar patterns of expression during early embryogenesis. Second, CRM and PCNA proteins co-localize in giant polytene nuclei. Third, *trans*-heterozygous crm^{7};$mus209^{B1}$ mutant males show strong enhancement of both anteroposterior and distal-proximal transformations. Together, these data suggest that these two proteins might be involved in common regulatory pathway(s). Since PCNA is essential for DNA replication, our data highlight possible interactions between *Pc*-G-mediated silencing and DNA replication in *Drosophila*. Evidence for an involvement of DNA replication in silencing come from studies of silencing at the mating-type loci in the budding yeast *Saccharomyces cerevisiae*. The silencer elements flanking the silent HMR and HML loci have the ability to allow autonomous replication of plasmids. Furthermore, proteins of the origin recognition complex (ORC) are required for both silencing and DNA replication (reviewed in Loo and Rine, 1995). Genes encoding proteins homologous to orc2p and orc5p have been cloned in *Drosophila* (Gossen et al., 1995), but their involvement in silencing has yet to be investigated in flies. Whether *crm* might be involved in some aspects of DNA replication is still unknown and will require the acquisition of a *crm* null allele. Nevertheless, preliminary data suggest that the CRM protein partially co-localizes with sites of DNA replication, visualized by BrdU staining in early embryos (Y. Y., unpublished data). Finally, both *crm* (this study) and *mus209* mutants (Henderson et al., 1994) suppress PEV, suggesting a role for CRM and PCNA in chromatin assembly and/or modification. Since heterochromatin is known to be late replicating (see Elgin, 1996, for a recent review), one can envisage that interactions between PCNA and *Pc*-G might regulate the timing of replication of repressed genes.

We thank the following colleagues for the gift of fly stocks, plasmids or antibodies: D. Henderson for the *mus209* flies, A. Preiss and D. Maier for anti-H antibody, B. Judd for the Df(1)X22 deficiency, C. Wilson for the genomic DNA library, L. Kauvar for the embryonic cDNA library, P. Fisher for polyclonal anti-PCNA antibody, M. Shannon for crm^{7} flies, R. Paro for anti-PC antibody, M. Goldberg for genomic clones, J. Montagne for baculovirus-*Antp* and baculovirus-*crm* constructs. We acknowledge the expert technical assistance of U. Kloter, S. Pfister and P. Baumgarnter. This work was supported by grants from the Schweizerischer Nationalfouds and the Kantons of Basel, the European Molecular Biology Organization (to F. G. and B. B.) and the Human Frontier Science Program (to F. G.).

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(Accepted 30 June 1997)