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The 190 kDa centrosome-associated protein of *Drosophila melanogaster* contains four zinc finger motifs and binds to specific sites on polytene chromosomes

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SUMMARY

Microinjection of a bacterially expressed, TRITC labelled fragment of the centrosome-associated protein CP190 of *Drosophila melanogaster*, into syncytial *Drosophila* embryos, shows it to associate with the centrosomes during mitosis, and to relocate to chromatin during interphase. Indirect immunofluorescence staining of salivary gland chromosomes of third instar *Drosophila* larvae, with antibodies specific to CP190, indicate that the protein is associated with a large number of loci on these interphase polytene chromosomes. The 190 kDa CP190 protein is encoded by a 4.1 kb transcript with a single, long open reading frame specifying a polypeptide of 1,096 amino acids, with a molecular mass of 120 kDa, and an isoelectric point of 4.5. The central region of the predicted amino acid sequence of the CP190 protein contains four $CysX_2CysX_{12}HisX_4His$ zinc-finger motifs which are similar to those described for several well characterised DNA binding proteins. The data suggest that the function of CP190 involves cell cycle dependent associations with both the centrosome, and with specific chromosomal loci.

Key words: centrosome, zinc finger protein, chromatin, polytene chromosome, *Drosophila*

INTRODUCTION

The key role played by the centrosome in coordinating the cytoskeletal changes that occur during the cell cycle, has been generally accepted since the organelle was first defined (see Wilson, 1896, for a review of the early descriptions of the centrosome). Extensive cytological analyses, using electron microscopy, have characterised centrosomal and centriolar structures from various organisms in considerable detail (Gould and Borisy, 1977; Bornens et al., 1987; Paintrand et al., 1992), and the development of effective methods for the isolation of centrosomes from tissue culture cells (Ring et al., 1980; Mitchison and Kirschner, 1984, 1986; Gosti-Testu et al., 1986) and calf thymus (Komesli et al., 1989), have allowed the identification of a spectrum of centrosome-associated proteins. Many other centrosome-associated components have been characterised by using either antisera or monoclonal antibodies, which recognise centrosomal antigens (reviewed by Kalt and Schliwa, 1993). However, a striking advance in our understanding of the centrosome has been achieved through genetic analysis of the fungus *Aspergillus nidulans* (Oakley and Oakley, 1989; Oakley et al., 1990). These studies identified γ-tubulin as a component of the spindle pole body essential for microtubule nucleation. Subsequent studies suggest that γ-tubulin is probably a ubiquitous component of centrosomes which plays an important role in cell cycle dependent microtubule nucleation (Zheng et al., 1991; Stearns et al., 1991; Joshi et al., 1992; Sunkel et al., 1995).

The numerous centrosome-associated proteins that have now been identified have been classified into four groups according to their pattern of localisation during the cell cycle (Kalt and Schliwa, 1993). The four groups defined by Kalt and Schliwa (1993) correspond to: (1) proteins located at the centrosome throughout the cell cycle; (2) centrosomal proteins detected only during mitosis; (3) proteins located at the centrosome during mitosis and elsewhere during interphase; and (4) other cellular proteins located at the centrosome. A well characterised example of a protein in group 3 is NuMA (also known as centrophilin, SP-H and SPN) (Tousson et al., 1991; Maekawa et al., 1991; Kallajoki et al., 1991), a 236 kDa protein found in vertebrate cells (Compton et al., 1992; Yang et al., 1992), which is a nuclear protein during interphase, but associates with the spindle poles when the nuclear envelope breaks down at the onset of mitosis. NuMA is thought to play a role in chromatin architecture, possibly as a component of the nuclear matrix (Compton and Cleveland, 1993, 1994; Zheng et

al., 1994), and it has been suggested that its association with the spindle pole occurs in order to ensure an equal distribution to daughter cells (Compton et al., 1992). However, it appears that NuMA may also play a role in the mitotic apparatus (Compton and Cleveland, 1993, 1994; Yang and Snyder, 1992), and it has recently been demonstrated that mutation of the predicted $P34^{cdc2}$ phosphorylation sites in NuMA disrupts the assembly of the mitotic spindle (Compton and Luo, 1995). Hence NuMA appears to fulfil a dual role in the cell, not only through its association with the spindle poles during mitosis, but also as a structural component of the nucleus during interphase.

Monoclonal antibody Bx63 recognises a centrosomal antigen of *Drosophila* (Frasch, 1985; Frasch et al., 1986), and the gene which encodes this centrosome-associated protein has been cloned (Whitfield et al., 1988). We have recently discovered that the centrosome-associated protein recognised by Bx63 is identical to DMAP190, a centrosome-associated protein which was originally identified by microtubule affinity chromatography (Kellogg et al., 1989; Kellogg and Alberts, 1992). By mutual agreement we have decided to adopt the name CP190 (for Centrosomal Protein of 190kDa), to identify this protein in future publications. CP190 would be classified as a group 3 centrosome-associated protein under the system of Kalt and Schliwa (1993), since like NuMA, it localises to nuclei during interphase (Frasch, 1985; Frasch et al., 1986; Whitfield et al., 1988). However, CP190 is distinct from NuMA in that its centrosome association is not dependent on the nucleated microtubule array (Raff et al., 1993). Whereas NuMA is a component of the spindle pole, CP190 is an integral component of the 'core' centrosome (which is our designation for the structure that remains after microtubules have been depolymerised). Other proteins which localise to the centrosome in the absence of microtubules, and thus may be considered as components of the 'core' centrosomes, include γ-tubulin and pericentrin. Pericentrin is a centrosomal component which has been identified in a wide variety of different species using autoimmune serum from a patient with scleroderma (Tufanelli et al., 1983; Calarco-Gillam et al., 1983; Clayton et al., 1985). It also shows microtubule-independent centrosomal-association, and Doxsey et al. (1994), have demonstrated that pericentrin plays a key role in the assembly and organisation of microtubule nucleating components of the centrosome.

Despite the growing list of centrosomal components which have been identified, our understanding of the molecular mechanisms which underlie microtubule nucleation and centrosome replication is still poor. It is clear that the centrosome undergoes dramatic cell cycle dependent changes in phosphorylation (Vorobjev and Chentzov, 1982; Rieder and Borisy, 1982; Vandré et al., 1984), structure (Robbins et al., 1968; Rieder and Borisy, 1982; Baron and Salisbury, 1988), nucleation capacity (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981), and function, and yet the molecular basis for these changes is almost completely unknown. Hence, a molecular analysis of the centrosome, and an investigation of its integration with other cell cycle events, is a major objective for the cell biologist. To this end, we are studying a centrosome-associated protein in *Drosophila melanogaster* (Whitfield et al., 1988). The protein, CP190, is unique in that it localises to the centrosome independently of nucleated microtubules (Raff et al., 1993), in a dramatic cell cycle dependent manner (Whitfield et al., 1988; Callaini and Riparbelli, 1990). In this paper, we demonstrate that the association of CP190 with the centrosome during mitosis, which is apparent from indirect immunofluorescence analyses, actually occurs in vivo, and we present evidence which suggests that, during interphase, the CP190 protein is associated with a large number of specific chromosomal sites.

MATERIALS AND METHODS

Isolation and DNA sequence analysis of CP190 cDNAs

The original clones which were shown to encode the CP190 centrosome-associated protein were derived from a λgt11 expression library containing randomly fragmented *Drosophila* genomic DNA (Whitfield et al., 1988). Approximately 50 ng of the 3.4 kb *Eco*RI insert from one of these clones (λcs3), was labelled with ^{32}P by the random priming method (Feinberg and Vogelstein, 1983, 1984), and used to screen a *Drosophila* cDNA library essentially as described by Sambrook et al. (1989). The cDNA library, made with mRNA from 0-4 hour embryos, was kindly provided by Dr Nick Brown (Brown and Kafatos, 1988). Five positive colonies were isolated from screening approximately 4×10^4 recombinant clones. All of the cDNA clones isolated had inserts in excess of 3 kb and showed similar restriction enzyme digest patterns (data not shown). One of the clones, pNBcs5 had an insert size of 4.1 kb, corresponding to the estimated size of the CP190 mRNA (Whitfield et al., 1988), and this clone was subsequently used for all further analyses. Both DNA strands of the pNBcs5 insert were sequenced by the chain termination method (Sanger et al., 1977), using Sequenase™ (Stratagene) according to the manufacturers instructions. A strategy of directed cloning, and Exonuclease III generated deletion (Henikoff, 1984), was employed in order to obtain the complete sequence of both strands. The CP190 sequence data was compiled and analysed using DNASTAR Lasergene software, and compared to the GenBank and EMBL databases through the HGRP computer system (Rysavy et al., 1992), using BLAST at the default settings (Altschul et al., 1990).

Expression, purification and TRITC labelling of CP190Δ125 protein

The CP190 cDNA contains a single *Nco*I restriction site in which the ATG codon is in-frame with the largest open reading frame. A restriction fragment of approximately 3.0 kb, running from this *Nco*I site to a *Bgl*II site just downstream of the end of the open reading frame, was subcloned into *Nco*I/*Bam*HI cut pWW1 (pWW1 is a modified version of the T7 expression vector pAR3038 (Studier and Moffat, 1986; Whitfield et al., 1990), in which the unique *Nde*I site has been replaced by an *Nco*I site). The resulting construct, pWWcp165 can express an unfused polypeptide (CP190Δ125) lacking only 125 N-terminal amino acids with respect to the predicted sequence of the CP190 protein. The CP190Δ125 protein was expressed in the *Escherichia coli* host BL21(DE3), essentially as described by Studier and Moffat (1986). Cells from induced cultures were resusupended in 0.1 of the initial culture volume of 50 mM Tris-HCl, pH 8.0, 1 mM Na_2EDTA, 0.1 M NaCl, 1 mM PMSF. Lysozyme was added to the cell suspension to a final concentration of 200 μg/ml, and the cells lysed by incubation at 0°C for 15 minutes followed by one or two freeze/thaw cycles. To remove the bulk of the DNA, $MgCl_2$ was added to 5 mM followed by 10 μg/ml of DNase I, and the lysate incubated for a further 10 minutes at 0°C. The lysate was then vigorously homogenised in a hand-held Dounce and centrifuged at 15,000 rpm (25,000***g*** (max)) for 15 minutes in a Sorvall SS34 rotor. At this stage, the majority (>90%), of the CP190 protein was in the form of inclusion granules in the insoluble pellet. The crude CP190Δ125 pellet was raised in 0.1 culture volumes

of buffer containing 8 M urea, 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl and recentrifuged at 15,000 rpm for 30 minutes to remove insoluble material. The supernatant was passed through a QMA MemSep^{tm}1000 cartridge (Waters), equilibrated in the same buffer. After washing the QMA cartridge with 10 sample volumes of 8 M urea, 50 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl, the CP190Δ125 protein was eluted with 1 sample volume of the same buffer containing 0.3 M NaCl. Purified protein was dialysed against 2× 5 l of 10 mM, Pipes, pH 7.0, 2 mM 2-mercaptoethanol, at 4°C over 24 hours, to remove the urea. A small amount of precipitated material, evident after the dialysis, was removed by low speed centrifugation and the soluble CP190Δ125 protein concentrated to approximately 5 mg/ml by centrifugation in a Microsep^{tm} centrifugal concentrator (Amicon). At this stage, on Coomassie Blue stained SDS-polyacrylamide gels, the preparation was estimated to consist of >95% intact CP190 protein. Purified CP190Δ125 protein was labelled with the fluorochrome tetramethyl rhodamine isothiocyanate (TRITC), essentially as described by Harlow and Lane (1988). The protein conjugate (now at an approximate concentration of 4 mg/ml), was stored frozen in 10 μl aliquots at –80°C.

Microinjection and confocal microscopy

Freshly laid *Drosophila* embryos (strain Oregon R), were manually dechorionated and arranged in an anterior to posterior orientation along a line of Magic^{tm} tape adhesive down the centre of a coverslip. After brief dehydration (approximately 5%), in a Petri dish containing silica gel, the coverslip was temporarily mounted (embryos upwards), on a standard slide and the embryos covered with a thin layer of Voltalef oil to prevent further dehydration. Microinjection needles were pulled from 25 μl glass capillaries (Drummond), with the aid of a Narashige PN-3 needle puller, and back-filled with TRITC-labelled CP190Δ125. For injection, the embryos were viewed on an inverted microscope under brightfield illumination, at low magnification. The needle tip was fractured on the edge of the coverslip to give an approximate tip diameter of 3 to 5 μm, and the embryos were microinjected at a point mid-way along their length. When the entire line of embryos (approximately 20 individuals), had been injected, the coverslip was inverted and mounted on a slide with double coverslip spacers. The embryos were initially localised, and suitable developmental stages selected, under bright field illumination on a Nikon Optiphot with a ×63 Plan-APOCHROMAT objective, and were subsequently scanned under epifluorescence illumination to identify those embryos that had been successfully injected. Embryos between nuclear cycle 9 and 12 were chosen for time series observation with the MRC600 (Bio-Rad) confocal attached to the same microscope. After selecting the correct focal plane under direct scan, images were recorded automatically every 60 seconds. Each image was derived from Kalman averaging of four scans (Erasmus, 1982). Near maximum apperture width and low-signal gain settings were necessary to obtain a reasonable image. Individual embryos were usually observed through one or two nuclear cycles, but occasional embryos were followed through four or five cycles of division.

Indirect immunostaining of embryos

Embryos were fixed and prepared for staining using a modification of the method of Mitchison and Sedat (1983). Embryos were dechorionated in 50% hypochlorite bleach, permeabilised and fixed in 5 ml of 10% formaldehyde in 0.1 M NaCl, 2 mM EGTA, 50 mM Pipes, pH 7.0, and 5 ml of heptane, and devitellinised in 5 ml of absolute methanol. The embryos were then equilibrated in phosphate buffered saline (PBS: 0.14 M NaCl, 3 mM KCl, 10 mM sodium phosphate buffer, pH 7.4), containing 0.1% Triton X-100. Approximately 200 embryos were transferred to 1.5 ml microfuge tubes containing 0.5 ml of 10% foetal calf serum (FCS) and 10 μg/ml RNAse A, in PBS. After overnight incubation at 4°C, the supernatant was removed and 0.5 ml of Rb188 antiserum diluted ×500 in 10% FCS/PBS containing 0.1%Triton X-100, was added to the embryos. The first antibody incubation was continued for 4 hours at room temperature before giving the embryos five 10 minute washes in PBS containing 0.1% Triton X-100. The embryos were then incubated at room temperature in darkness, with 0.5 ml of FITC-conjugated goat anti-rabbit Ig(H+L) (Jackson Laboratories), diluted ×500 in 10% FCS in PBS. The 5× 10 minute washing step was repeated with two further rinses in PBS alone, before mounting the embryos in Mowiol (Harlow and Lane, 1988), containing 0.1 μg/ml of propidium iodide and 2.5% *n*-propylgallate. Slides were viewed using a ×63 Plan-APOCHROMAT objective (NA 1.4), on a Zeiss Axioskop microscope with epifluorescence optics and appropriate filters for FITC and propidium iodide. Images were recorded digitally using a Hamamatsu C3077 CCD camera and Fluovision^{TM} software (Improvision). Digital images were normalised and arranged for publication using Adobe Photoshop^{TM} software.

Indirect immunostaining of polytene chromosomes

Salivary glands were dissected from third instar larvae in *Drosophila* Ringer's solution 182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2. The glands were transferred to a drop of 45% acetic acid, 3.7% formaldehyde on a siliconised coverslip and incubated for 2 to 3 minutes before mounting the coverslip on a clean slide and spreading the chromosomes by tapping the coverslip. Slides were frozen briefly in liquid nitrogen, the coverslips flipped off with a razor blade and the slides immediately plunged into PBS at room temperature. Chromosome preparations to be immunostained with the anti-DMAP60 antibody, required prefixation of the salivary glands in 3.7% formaldehyde in PBS for 45 seconds before transfer to 45% acetic acid, 3.7% formaldehyde for squashing. After 5 minutes in the PBS, the slides were removed and drained. Between 100 and 200 μl of antibody, appropriately diluted in 3% bovine serum albumin (BSA), in PBS, was then applied to the squash and the slide incubated in a humid chamber for 1 hour at room temperature. Slides were given 5× 5 minute washes in PBS before repeating the incubation with the appropriate second antibody (protected from the light), also diluted in 3% BSA in PBS. After a further 5 washes in PBS, the slide was briefly rinsed in dH_2O, drained and mounted in Mowiol containing 2.5% *n*-propylgallate and either 0.1 μg/ml propidium iodide or 0.5 μ/ml DAPI. Slides were examined on a Nikon Optiphot using a ×63 plan-APOCHROMAT objective with epifluorescence optics. Confocal images were recorded on an MRC600 with a Krypton/Argon laser. Single channel collection was used, with exciter filters for lines at 488 nm (FITC), and 568 nm (TRITC and propidium iodide), in order to prevent any bleed through of signal between channels. Images were printed on a SONY UP-5000P video printer.

Electrophoresis and western blotting

Samples of total proteins from salivary glands of third instar larvae were resolved by electrophoresis on either 7.5% or 12% SDS-polyacrylamide gels (Laemmli, 1970). The proteins were electrophoretically transferred to nitrocellulose filters using a mini-transblot apparatus (Life Technologies), according to the method of Towbin et al. (1979), except that methanol was not added to the transfer buffer. Filters were blocked overnight at 4°C in 5% powdered milk in PBS, and incubated at room temperature with first antibody diluted in 5% milk/PBS for 2 hours at room temperature. After 3 fifteen minute washes in PBS containing 0.1% Triton X-100, the filters were incubated for a further 2 hours in peroxidase-conjugated second antibody diluted in 5% milk/PBS. At the end of the second incubation, the filters were washed as before and the signal developed using Amersham ECL reagents according to the manufacturers instructions. Filters were exposed to Fuji RX film for 10 minutes and developed automatically in an AGFA Curix 60.

RESULTS

CP190 is a nuclear protein which associates with the centrosome during mitosis

Previous investigations of the localisation of CP190 protein in syncytial *Drosophila* embryos, have shown that the CP190

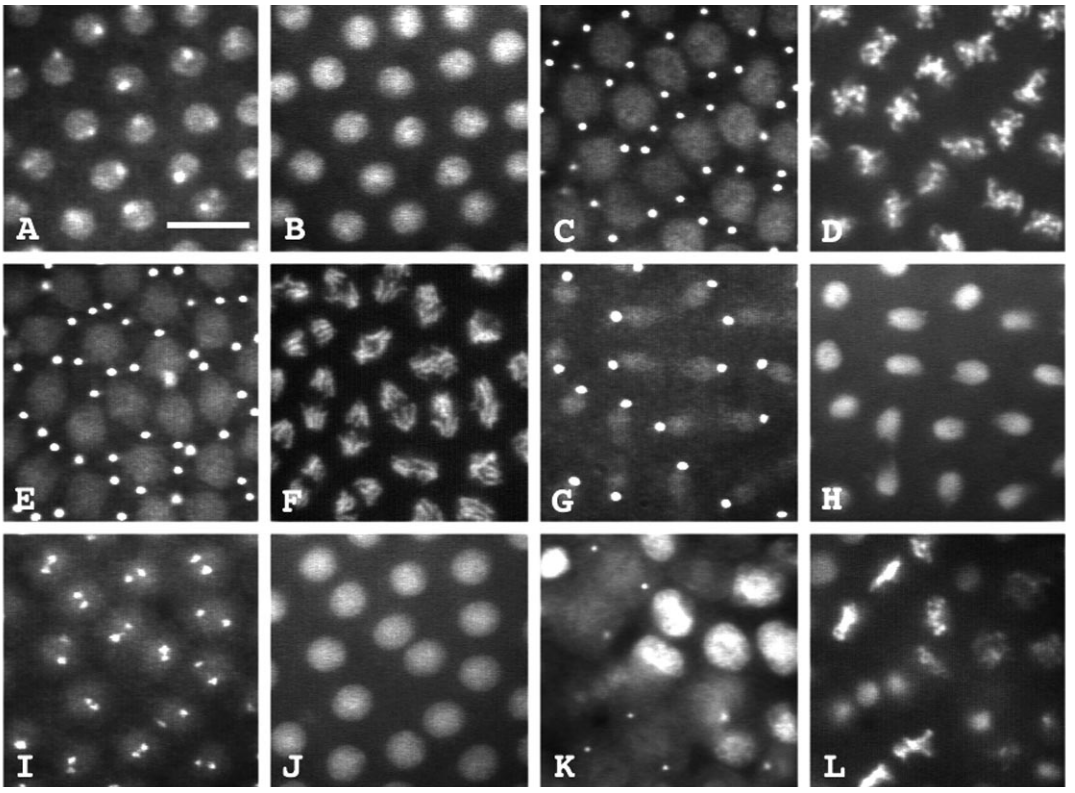


Fig. 1. Indirect immunofluorescence staining of *Drosophila* embryos with anti-CP190 polyclonal antibody Rb188. Embryos were incubated with Rb188 followed by FITC-conjugated anti-rabbit Ig and the DNA fluorochrome DAPI. (A,C,E,G,I,K) The Rb188 staining at various nuclear cycle stages; (B,D,F,H,J,L) DAPI staining of the corresponding fields. Bar, 10 μm. During interphase (A), the centrosomes are clearly stained and a more diffuse staining is evident in the nucleus beneath each centrosome. During metaphase (C), bright anti-centrosomal staining is visible at both poles and a diffusely stained zone remains over the region occupied by the spindle. The anti-CP190 staining pattern remains very similar during early anaphase (E), but during anaphase B and early telophase (G), the diffuse staining relocalises to the chromatin. (I) Rb188 staining of centrosomes as they divide and begin to migrate to opposite poles at early prophase and, once again, diffuse staining of the chromatin is evident. After cellularization, centrosomal staining is visible at either pole of the mitotic figures in K. However, in the same panel, it is clear that the interphase nuclei are not associated with stained centrosomes.

protein is associated with the centrosome throughout the nuclear division cycle (Whitfield et al., 1988; Callaini and Riparbelli, 1990). During mitosis, not only is the centrosome intensely stained, but the region corresponding to the spindle shows elevated levels of the CP190 protein (Fig. 1C and E). Furthermore, during nuclear cycles 9 to 14, it is clear that CP190 is also present in the interphase nuclei (Fig. 1A and I). However, after cellularisation of the embryo at nuclear cycle 14, it appears that CP190 is exclusively nuclear during interphase and is only centrosome-associated in mitotic domains (Fig. 1K).

A truncated form of CP190, can undergo several cycles of nuclear/centrosomal cycling when microinjected into syncytial embryos

By exploiting a unique *Nco*I site containing an in-frame ATG located only 375 nucleotides downstream of the presumptive translational initiation codon in the original CP190 cDNA, we were able to express an extensive domain of the CP190 protein in *E. coli*. The unfused polypeptide (CP190Δ125), expressed from the *Nco*I site, only differs from the predicted full-length CP190 protein by the truncation of 125 amino acid residues from the NH_2-terminal. Purified CP190Δ125 was labelled with TRITC and introduced into syncytial *Drosophila* embryos by microinjection. Observation of the fate of TRITC-labelled CP190Δ125, by time-lapse confocal microscopy, reveals that its distribution during subsequent cycles of nuclear division, is almost identical to that observed by indirect immunofluor-escence localisation of the native protein in fixed embryos. Within 5 minutes of the injection of TRITC-labelled CP190Δ125, a marked accumulation of the fluorescent protein can be seen in interphase nuclei. Fig. 2 shows the distribution of TRITC labelled CP190Δ125 at various stages through a single nuclear division cycle (see legend for the precise timing of each stage).

As interphase proceeds, the fluorescent nuclei increase in size, before a gradual loss of staining at what we assume to be the onset of prophase (Fig. 2A). The disappearance of nuclear fluorescence coincides with the appearance of bright dots of staining on either side of the fading nucleus (Fig. 2B), precisely where the poles of the mitotic spindle would be expected to lie. This centrosomal fluorescence remains strong during metaphase (Fig. 2C and D), and apparently intensifies as the poles move apart at anaphase B (Fig. 2E). As the nuclei reform, the centrosomal fluorescence is lost (Fig. 2F and G), and the fluorescence becomes exclusively nuclear again (Fig. 2H). It should be noted that after microinjection, TRITC labelled CP190Δ125 is only visible at the centrosome during mitosis, whereas during interphase, fluorescent centrosomes cannot be detected at the expected position above the nucleus. In this respect, the distribution of TRITC labelled CP190Δ125 in vivo is different from that observed for CP190 by indirect immunofluorescence of fixed syncytial embryos, where although CP190 is strongly detected in interphase nuclei, some centrosome staining is also seen throughout the nuclear division cycle. Control microinjections of TRITC-labelled bovine serum albumin showed no specific nuclear or centrosomal accumulation (data not shown).

The CP190 protein has 3 conserved zinc-finger motifs

The original CP190 clone, λcs3, was identified by screening a

Drosophila genomic DNA λgt11 expression library with monoclonal antibody Bx63 (Whitfield et al., 1988). The *Eco*RI fragment of genomic DNA from λcs3 was used as a probe to screen a 0-4 hours *Drosophila* embryo cDNA library (Brown and Kafatos, 1988). A number of cDNA clones were identified, one of which (pNBcs5), contained a 4.1 kb insert (Fig. 3A), which corresponds closely to the estimated size for the CP190 mRNA of 4.2 kb, and is probably a full-length cDNA. Sequence analysis of the CP190 cDNA reveals a single, long open reading frame in which the upstream flanking sequence of the first in-frame AUG codon at nucleotide 188 (CAAUAUG), conforms reasonably closely to the consensus sequence of (C/A)AA(A/C)AUG for an initiation codon in *Drosophila* (Cavener, 1987). The predicted sequence of 1,096 amino acid residues encoded by this open reading frame would produce a protein with an isoelectric point of 4.5 and a molecular mass of 120 kDa. This is considerably less than the apparent molecular mass of CP190 (190 kDa), estimated by comparison to standard proteins on SDS-polyacrylamide gels. Although the discrepancy in size could be due to post translational modification of the protein, it is more probably due to aberrant electrophoretic mobility, since expression of truncated portions of the CP190 cDNA also produces polypeptides with mobilities proportionally greater than that predicted for the size of the open reading frame expressed (not shown).

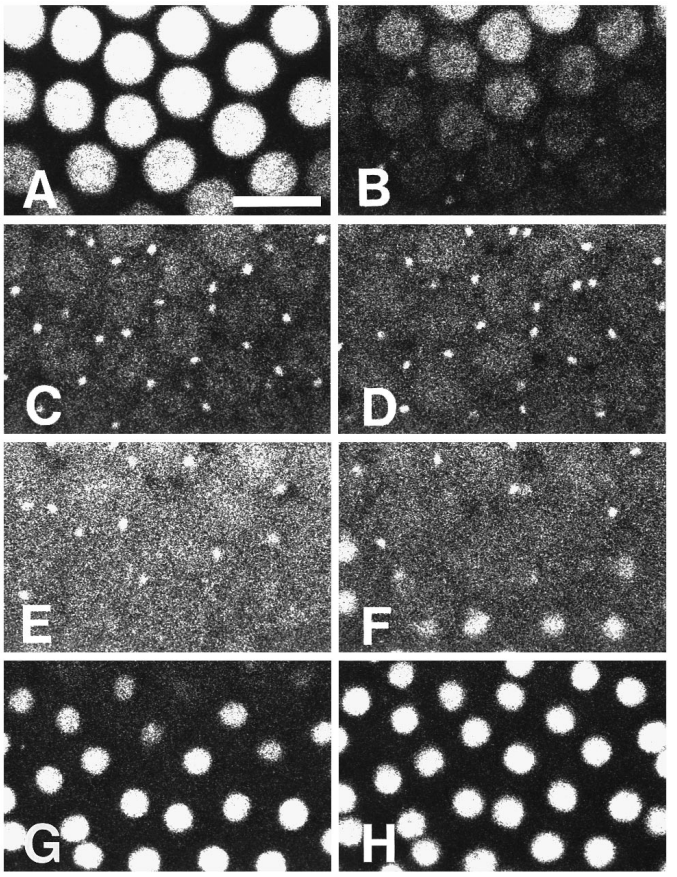


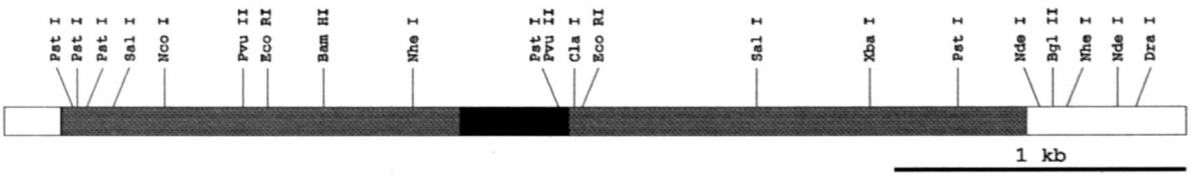
Fig. 2. (A to H) A timed series of confocal images of the distribution of TRITC-labelled CP190Δ125, after microinjection into a syncytial *Drosophila* embryo. Bar, 10 μm. In the first image (A; reference time point *t*=0), the labelled protein is exclusively localised in the interphase nuclei. At prophase, in B (*t*=2 minutes), nuclear staining is dispersing and the labelled protein is accumulating at the centrosomes in a parasynchronous wave from the bottom to the top of the field. C (*t*=4 minutes) and D (*t*=6 minutes) illustrate the distribution of the labelled CP190Δ125 during metaphase, and E (*t*=8 minutes) and F (*t*=9 minutes) during anaphase and early telophase, respectively. In F and G (*t*=11 minutes) the centrosomal staining is disappearing and nuclear staining reappearing, and by H (*t*=13 minutes), the labelled CP190Δ125 is localised exclusively in the interphase nuclei once more.

Comparison of the predicted amino acid sequence of the CP190 cDNA to the SWISSPROT database using the BLAST programme (Altschul et al., 1990; Rysavy et al., 1992), identifies two classes of proteins with which it shares similarities. The first class are proteins such as neurofilaments, myosin heavy-chain and MAP-2, with which it shares a low level of amino acid identity (<15%), over a considerable proportion of its length. Although most of the proteins identified in this class possess extensive coiled-coil structural motifs (Conway and Parry, 1991), analysis of the amino acid sequence of CP190 using the COILS 2.1 programme (Lupas et al., 1991), reveals only three short regions with any significant probability of forming coiled-coil structure. The second class of proteins identified in the database were characterised by the possession of $CysX_2CysX_{12}HisX_4His$ zinc-finger motifs (Klug and Rhodes, 1987). The CP190 protein has a cluster of 4 zinc-fingers which occur roughly in the middle of the predicted protein (dark-shaded region of Fig. 3A; grey boxed sequence in Fig. 3B). The first finger is rather degenerate, having a finger loop of 17 amino acids rather than the usual 12, but the remaining 3 fingers show between 25 and 32% identity with the zinc-finger domains of a number of well characterised transcription factors including *serendipity*-β and δ, *tramtrack* and *krüppel* (Vincent et al., 1985; Payre and Vincent, 1991; Harrison and Travers, 1990; Fairall et al., 1992; Jäckle et al., 1985; Licht et al., 1990). Fig. 4A shows an alignment of zinc-fingers 2, 3 and 4 from CP190, with each other, the dashes represent gaps introduced to compensate for the variable spacing between the fingers. Apart from the conserved positions of the cysteine and histidine residues, a number of other positions are important for the structural integrity of the C_2H_2 zinc finger (Rosenfeld and Margalit, 1993). In bona fide DNA binding zinc finger motifs the best conserved residue is found at position 4 in the finger loop, which is generally an aromatic or aliphatic amino acid. Although the second finger loop in CP190 does not fit this pattern (it has a serine residue at this position), fingers 3 and 4 do conform, with tyrosine and phenylalanine residues respectively. Furthermore, fingers 2, 3 and 4 have a conserved leucine at position 10 within the loop, which is also a feature common to many of those zinc-finger proteins shown to have sequence-specific DNA-binding activity. Fig. 4B shows an alignment of zinc-fingers 2, 3 and 4 from CP190, with fingers 1, 2 and 3 from the *serendipity*-δ transcription factor (Vincent et al., 1985): the two sequences share >30% identity over the range shown. The zinc-fingers of the CP190 protein also show a similar degree of identity with several zinc-finger proteins from species other than *Drosophila* (not shown).

CP190 is associated with numerous sites on polytene chromosomes

In order to determine whether the CP190 protein interacts with specific chromosomal loci, we carried out indirect immunoflu-

A



B

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CCCCCCCCCCGGAATGGAAAAGAATATGGCGACCTGACTCTGGAAAAAAATTATAATTTG<60
TCCGGCAAAAGTAGTGTGCAATTCAAAGAAATTTGCGCCCTGAAGCCAGAGCAAGCGAAA<120
CCACGGGGAGGCTAACGATAAGGATACGGAAACGGAGAGCACGGCCACTAGCCAAGCACA<180
      M  G  E  V  K  S  V  K  V  D  N  W  G  V  F  F  L  Q <18
CAGCAATATGGGTGAAGTCAAGTCCGTGAAAGTGGACAACTGGGGAGTCTTCTTCCTGCA<240
  K  L  Q  N  F  F  N  K  T  D  Y  C  D  L  T  L  Q  F  R  D <38
GAAGCTGCAGAACTTCTTTAATAAAACGGATTACTGCGATTTGACGCTGCAGTTTCGGGA<300
  N  S  Q  L  K  V  H  R  L  V  L  S  A  C  T  D  Y  F  N  V <58
CAATTCACAGCTAAAGGTACACCGTCTGGTGCTCAGCGCCTGCACCGACTACTTCAATGT<360
  L  E  Q  T  C  E  I  V  D  D  A  L  I  M  P  N  E  F  Q  A <78
ACTGGAGCAGACCTGCGAGATCGTCGACGATGCCCTCATCATGCCCAACGAGTTCCAGGC<420
  D  V  V  V  P  I  V  N  F  M  Y  T  G  T  L  E  F  E  L  K <98
GGACGTGGTGGTGCCCATTGTCAACTTCATGTACACAGGCACCCTGGAGTTTGAGCTTAA<480
  M  Y  G  K  L  L  R  T  A  K  E  M  N  M  T  V  L  L  K  L <118
AATGTACGGCAAGCTGTTGCGCACCGCCAAGGAAATGAACATGACGGTGCTGTTGAAGCT<540
  L  E  A  H  R  R  T  M  E  N  V  N  R  Q  Q  R  P  P  S  P <138
GCTAGAGGCGCACCGTCGCACCATGGAGAACGTTAATCGCCAGCAAAGGCCGCCTAGTCC<600
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orescence staining of polytene chromosomes from salivary glands of third instar larvae, with antibodies specific to the CP190 protein. A considerable number of fluorescent sites along the entire length of the polytene set are stained. Fig. 5 shows a region at the end of the X chromosome immunostained with either the Rb188 anti-CP190 polyclonal (Fig. 5A), or the Bx63 monoclonal antibody (Fig. 5B). Frasch (1985) had previously reported that the Bx63 monoclonal antibody recognised numerous polytene loci, but at that time it was not possible to determine whether they corresponded to the presence of the 190 kDa polypeptide or an additional 68 kDa polypeptide identified by the Bx63 antibody on western blots. However, because the Rb188 antiserum recognises the 190 kDa but not the 68 kDa polypeptide (i.e. the epitope of the Bx63 monoclonal antibody is not recognised by Rb188 (Whitfield et al., 1988)), we have been able to resolve this question. The merged image in Fig. 5C clearly demonstrates that the sites recognised by the Bx63 monoclonal are identical to those recognised by the Rb188 antiserum, this suggests that the pattern of staining must be due to the presence of CP190 protein at these sites, and not the result of some fortuitous cross-reaction.

The original interpretation of the Bx63/polytene chromosome staining pattern put forward by Frasch (1985), was that the sites identified essentially corresponded to the chromosomal interbands. Fig. 6 shows an immunofluorescence micrograph of a region at the tip of the X-chromosome stained with the Rb188 antiserum (Fig. 6A), in comparison with the same region stained with propidium iodide to identify the DNA (Fig. 6B). Merging these two images (Fig. 6C), reveals that the distribution of the CP190 protein does not merely mirror either the band or interband pattern, but appears to localise to the band/interband boundaries. Furthermore, the obvious puff at 2B5.6 appears to contain very little CP190 protein, and that which is present seems to be associated with the edges of discrete regions of condensed chromatin. Ribonuclease treatment of the polytene chromosome preparations prior to immunostaining has no apparent effect on the banding pattern observed (data not shown). This result suggests that the CP190 protein is associated with chromatin rather than ribonucleoprotein, although since treatment with ribonuclease could leave ribonucleoproteins precipitated in situ, we cannot exclude the possibility that CP190 is an RNA binding protein.

Fig. 3. Restriction map (A), and nucleotide sequence (B), of the *Drosophila* CP190 cDNA (pNBcs5). The nucleotide sequence of the CP190 cDNA has an open reading frame encoding a polypeptide of 1,096 amino acid residues. The derived amino acid sequence is shown in single letter code above the nucleotide sequence. The translation of the longest open reading frame is shown from an ATG codon with flanking bases which conform closely to the consensus sequences for translational start sites in *Drosophila* (Cavener, 1987). The boxed sections of amino acid sequence correspond to the four putative C_2H_2 zinc fingers, and the best candidate polyadenylation signal is underlined. On the restriction map, the position of the open reading frame is indicated by the light grey shading and the black box indicates the extent of the zinc finger domain. The regions which could potentially form coiled-coil structures as predicted by the COILS 2.1 programme of Lupas et al. (1991), are residues 750 to 777 (P=0.875), and residues 888 to 915 (P=0.675). The probabilities in parenthesis are derived using the MTIDK matrix and a 28 residue window.

Fig. 4. (A) An internal alignment of the three conserved $CysX_2CysX_{12}HisX_4His$ zinc fingers from the CP190 protein, between amino acid residues 507 and 601 (fingers 2, 3 and 4). The regions of amino acid identity are indicated by shaded boxes, and the dashes represent spaces introduced in order to maintain the finger alignment. Note the conservation of a leucine residue at the tenth position within the finger-loop. (B) An alignment of the same three fingers from CP190 with fingers 1, 2 and 3 of the transcription factor encoded by *serendipity*-δ (Vincent et al., 1985). The two amino acid sequences show over 30% identity over the range shown. Note that fingers 2 and 3 of Serendipity-δ are $CysX_2CysX_{12}HisX_3His$ zinc fingers, and dashes have been introduced between the histidine residues in order to maintain the alignment.

CP190 binds to chromatin in association with another centrosomal protein

Using low affinity anti-CP190 (DMAP190) antibodies, Kellogg and Alberts (1992), identified additional proteins In *Drosophila* embryos which were also centrosome-associated. One of these proteins, CP60 (previously referred to as DMAP60), showed a distribution during the nuclear division cycle which was very similar to that of CP190. Raff et al. (1993), subsequently demonstrated that γ-tubulin is also present in eluates from anti-CP190 antibody columns. In the light of these observations, we wondered whether the association between CP190 and CP60 was confined to the cytoplasm, or whether it was maintained within the nucleus. Fig. 7 shows an immunofluorescence micrograph of a region at the tip of the X-chromosome immunostained with either Rb188 (Fig. 7A), or affinity purified antibodies specific to CP60 (kindly provided by K. Schneider) (Fig. 7B). Merging of theses two images (Fig. 7C), demonstrates that there is extensive (but not total), co-localisation of CP190 and CP60. Fig. 8 is a western blot of total protein from isolated salivary glands, immunostained with either Rb188 (Fig. 8A), or anti-CP60 (Fig. 8B), showing clearly that both CP190 and CP60 proteins are present in these preparations. From these data, it would seem reasonable to assume that CP60 is associating with most, if not all of the polytene sites which are binding CP190.

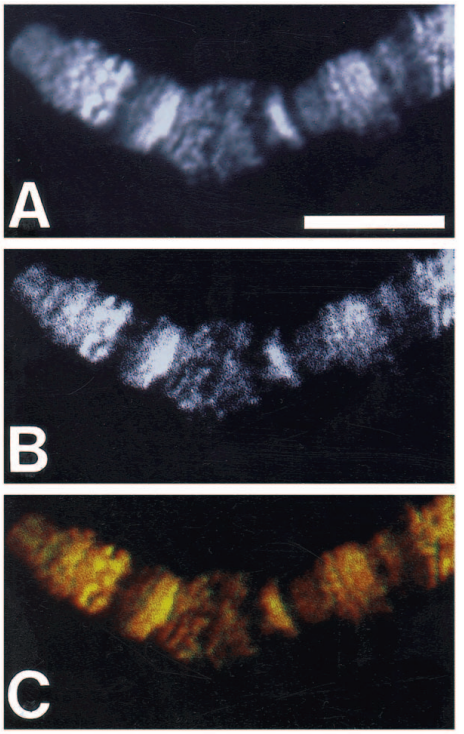

Fig. 5. Indirect immunofluorescence staining of salivary gland polytene chromosomes with either a polyclonal anti-CP190 antiserum (Rb188) and FITC-conjugated goat anti-rabbit Ig(H+L) second antibody (A), or with the Bx63 monoclonal antibody and TRITC-conjugated goat anti-mouse Ig(H+L) second antibody (B). Both panels show confocal images of the tip of the X chromosome. Note that essentially all the stained loci appear as yellow bands in the merged image (C), indicating that Rb188 and Bx63 are both recognising the same chromosomal sites. Bar, 10 μm.

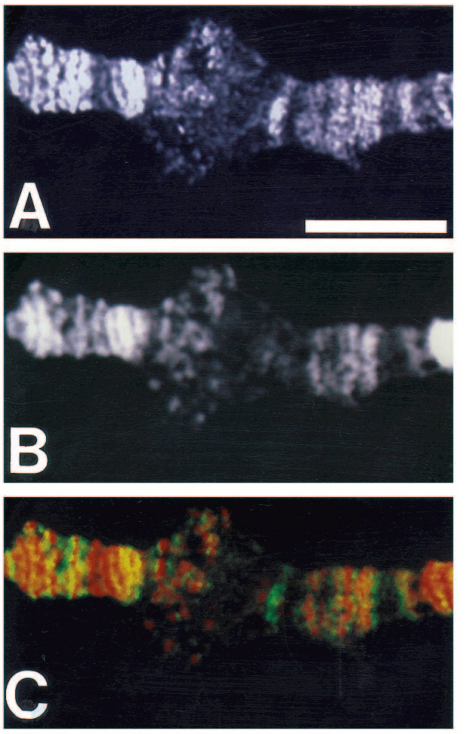

Fig. 6. Indirect immunofluorescence staining of salivary gland polytene chromosomes with either Rb188 and FITC-conjugated goat anti-rabbit Ig(H+L) second antibody (A), or with propidium iodide (B). Both panels show confocal images of the tip of the X chromosome. Note that in the merged image (C) the majority of the stained loci appear as either green bands (Rb188 stained), or red bands (propidium iodide stained), and only a few regions of overlapping staining (yellow bands), are evident. Bar, 10 μm.

Similar experiments using antisera specific to *Drosophila* γ-tubulin, indicate that despite its association with CP190 and CP60 in embryo extracts, γ-tubulin is not associated with either of these proteins in the salivary gland nuclei.

DISCUSSION

The nuclear division cycles during the syncytial phase of *Drosophila* development are characterised by their rapidity; they essentially lack both G_1 and G_2 phases, and consequently, a single nuclear cycle may take less than 10 minutes. In order to facilitate such rapid divisions, the components required for this stage of development have to be maternally provided. CP190 protein is maternally provided as protein, since semi-quantitative western blotting experiments suggest that its abundance does not change during syncytial development, and the CP190 protein is abundant in unfertilised eggs (data not shown). This interpretation implies the existence of a pool of cytoplasmic CP190 protein in the syncytium, from which it can be recruited to both nuclei and centrosomes as divisions proceed. The continuity of centrosome staining during syncytial development suggests that cytoplasmic CP190 protein must be continuously available throughout this period, in order to associate with the centrosome during interphase. However, in the absence of any significant levels of CP190 protein synthesis, the cytoplasmic pool of CP190 at interphase would be depleted as the nuclear/cytoplasmic ratio increased with each successive division. Hence, when cellularisation occurs at cycle 14, it may be that all of the maternal pool of CP190 protein is sequestered into nuclei, leaving no cytoplasmic CP190 to associate with the centrosomes. This explanation is consistent with the observation that from cellularisation onwards, centrosome-associated CP190 is only observed in cells which are undergoing mitosis.

The pattern of CP190 distribution observed in fixed preparations of embryos is largely confirmed by the behaviour of microinjected CP190Δ125 in vivo. Although this bacterially expressed protein lacks 125 N-terminal amino acids, and has been through a cycle of denaturation/renaturation during its purification, it must still retain a functional nuclear localisation sequence and a motif which mediates its association with the centrosome. Experiments involving the microinjection of a variety of CP190 fusion protein constructs indicate that the nuclear localisation and centrosome association properties of CP190 are located in separate domains of the protein (Oegemaet al., 1995).

The cell cycle specific distribution pattern of CP190 raises a number of questions with respect to its function. In particular, is the major role of CP190 enacted at the centrosome or in

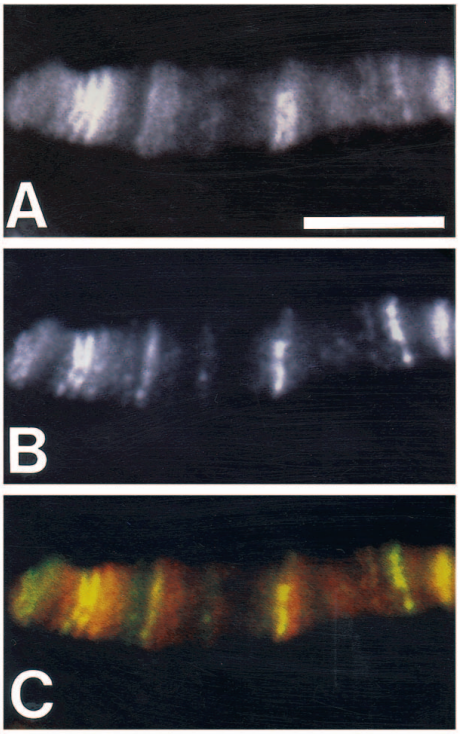

Fig. 7. Indirect immunofluorescence staining of salivary gland polytene chromosomes with either affinity purified anti-CP60 antiserum and FITC-conjugated goat anti-rabbit Ig(H+L) second antibody (A), or with the Bx63 monoclonal antibody and TRITC-conjugated goat anti-mouse Ig(H+L) second antibody (B). Both panels show confocal images of the tip of the X chromosome. Note that in the merged image (C) many, but not all of the strongly stained loci are common to both antisera (yellow bands). Bar, 10 μm.

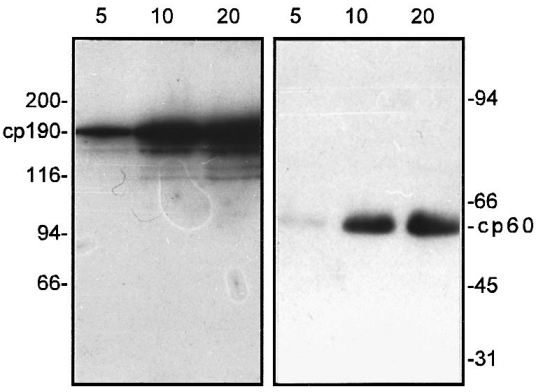

Fig. 8. Western blot of total protein extract from salivary glands of *Drosophila* third instar larvae, immunostained with either Rb188 or affinity purified anti-CP60. Salivary gland proteins were resolved on either 7.5% polyacrylamide (left panel) or 12% polyacrylamide (right panel) SDS gels and transferred to nitrocellulose filters. The filters were stained with the Rb188 anti-CP190 antiserum (left panel), or with affinity purified anti-CP60 antiserum (right panel). In both cases a peroxidase-conjugated goat anti-rabbit second antibody and enhanced chemiluminescence reactions (Amersham-ECL) were used to obtain the signal. The three tracks in each panel correspond to total protein from 5, 10 and 20 pairs of salivary glands, as indicated above each track. Positions of M_r standards are indicated ($\times 10^{-3}$).

the nucleus, or are both associations equally important? Although there is no direct evidence to answer these questions, a number of observations can be made which relate to the nuclear role of the protein. Many proteins with C_2H_2 zinc finger motifs have been shown to be transcription factors: the zinc fingers mediating the recognition of specific DNA sequences. CP190 shows significant similarity to several different C_2H_2 zinc finger proteins including *serendipity* β and *serendipity* δ (Vincent et al., 1985). Both *sry* β and *sry* δ encode C_2H_2 proteins which bind in vivo to distinct sites on polytene chromosomes (Payre and Vincent, 1991), and the *serendipity* δ zinc finger protein has recently been shown to regulate the transcription of the *bicoid* gene (Payre et al., 1994). However, CP190 associates with a very much larger set of sites on polytene chromosomes than either of the proteins encoded by *serendipity* β or δ. Furthermore, the relative abundance of CP190 in the early embryo is very high, suggesting that it is not a typical transcription factor but may play a role in the regulation of chromatin structure. This kind of structural/regulatory role is exemplified by a variety of non-histone chromosomal proteins which have recently been identified in *Drosophila*. For example, the *polycomb* group genes encode a number of proteins which appear to be responsible for maintaining the repressed state of homeotic genes during development, by maintenance of chromatin in a compact and therefore transcriptionally inactive state (Franke et al., 1992; Messmer et al., 1992). In contrast, the protein encoded by *E*(*var*)3-93D, which associates with a large number of polytene chromosome sites, is thought to be involved in establishing or maintaining an open chromatin conformation (Dorn et al., 1993), and Champlin et al. (1991) have identified a protein (B52), which is associated with the boundaries of transcriptionally active chromatin. Another protein, encoded by the *suppressor of Hairy wing* gene, has a domain containing seven zinc fingers, which is required for the recognition of a specific DNA sequence located in the 5′ untranslated region of the *gypsy* transposon (Spana et al., 1988). The *su*(*Hw*) protein is thought to act as a negative regulator of enhancer function, by interacting with enhancer-bound transcription factors via a leucine zipper domain (Harrison et al., 1993). The product of the *Trithorax-like* gene, GAGA factor, is another zinc finger protein that appears to be associated with large numbers of chromosomal loci (Tsukiyama et al., 1994). *Trl* appears to exert a regulatory role on the expression of several homeotic genes, and mutations in *Trl* also enhance position effect variegation, suggesting a role for GAGA factor in the modulation of chromatin structure (Farkas et al., 1994). As its name suggests, GAGA factor associates with purine rich motifs in the regulatory regions of several genes (Gilmour et al., 1989; Soeller et al., 1993; O'Donnell and Wensink, 1994), but unlike CP190, it also appears to bind to specific regions of heterochromatin (Raff et al., 1995).

Which, if any, of these classes of chromatin associated proteins would include the CP190 protein it is presently impossible to judge. However, we are currently investigating the nature of the specificity of the binding of CP190 to chromatin, in the hope that the identity of the CP190 target will reveal its function in the nucleus.

The role of the CP190 protein in association with the cen-

trosome remains similarly elusive. In experiments involving the microinjection of TRITC-labelled CP190Δ125, we have been able to follow the localisation of the tagged protein through up to four cycles of nuclear division. Aside from a minor increase in the length of the nuclear division cycle, the presence of the CP190Δ125 does not appear to have any adverse effects upon the division process, we are currently investigating whether the microinjection of other truncated or rearranged versions of the CP190 protein have any obvious phenotypic effects. One observation which may be informative with respect to the centrosomal role of the CP190 protein is that, in the acentriolar *Drosophila* cell line 1182, the Bx63 monoclonal antibody does not recognise centrosomes at any stage of the cell cycle (Debec and Abbadie, 1989). Despite the absence of centrosomal staining, the CP190 protein is present at normal levels in the 1182 cell line, but is apparently dispersed through the cytoplasm during mitosis rather than being concentrated at the spindle poles. This observation would suggest that the presence of the CP190 protein at the centrosome is not necessary for the proper functioning of the mitotic spindle. Furthermore, although the immunolocalisation of CP190 is normally pericentriolar, and insufficiently discrete to be considered as a centriolar component, the correlation between the absence of centrosomal CP190 and the acentriolar state of the 1182 cell line, may indicate an association with the centriole. In order to answer this and other questions, we are currently trying to identify mutations at the CP190 locus which will facilitate genetic analysis of the function of this protein.

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