Isolation of Chromosome-associated Proteins from *Drosophila melanogaster* That Bind a Human Centromeric DNA Sequence

Maria C. Avides* and Claudio E. Sunkel*‡

*Centro de Citologia Experimental da Universidade do Porto, 4100 Porto, Portugal; and ‡Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4000 Porto, Portugal

Abstract. The molecular mechanism involved in packaging centromeric heterochromatin is still poorly understood. CENP-B, a centromeric protein present in human cells, is thought to be involved in this process. This is a DNA-binding protein that localizes to the central domain of the centromere of human and mouse chromosomes due to its association with the 17-bp CENP-B box sequence. We have designed a biochemical approach to search for functional homologues of CENP-B in *Drosophila melanogaster*. This strategy relies upon the use of DNA fragments containing the CENP-B box to identify proteins that specifically bind this sequence. Three polypeptides were isolated by nuclear protein extraction, followed by sequential ion exchange columns and DNA affinity chromatography. All three proteins are present in the complex formed after gel retardation with the human alphoid satellite DNA that contains the CENP-B box. Footprinting analysis reveals that the complex occupies both strands of the CENP-B box, although it is still unclear which of the polypeptides actually makes contact with the DNA. Localization of fluorescein-labeled proteins after microinjection into early *Drosophila* embryos shows that they associate with condensed chromosomes. Immunostaining of embryos with a polyclonal serum made against all three polypeptides also shows chromosomal localization throughout mitosis. During metaphase and anaphase the antigens appear to localize preferentially to centromeric heterochromatin. Immunostaining of neuroblasts chromosome spreads confirmed these results, though some staining of chromosomal arms is also observed. The data strongly suggests that the polypeptides we have identified are chromosomal binding proteins that accumulate mainly at the centromeric heterochromatin. Furthermore, DNA binding assays clearly indicate that they have a high specific affinity for the human CENP-B box. This would suggest that at least one of the three proteins isolated might be a functional homologue of the human CENP-B.

In the interphasic nuclei of Eukaryotic cells a fiber of DNA measuring roughly up to 2 m when extended folds so as to occupy a sphere of barely 10 μm diameter. To achieve this, the DNA fiber binds to proteins and assumes several levels of compaction. The basic organization of Eukaryotic chromatin within the nucleus is thought to involve first the arrangement of short (145 bp) stretches of DNA wrapped around each nucleosome core particle. Short 30–50-bp linker DNA segments separate adjacent nucleosomes (McGhee and Felsenfeld, 1989). Secondly this 10-nm nucleosomal fiber is coiled into a solenoid-like structure giving rise to the 30-nm chromatin fiber (Woodcock et al., 1984; Widom and Klug, 1985; Felsenfeld and McGhee, 1986). The 30-nm chromatin fiber is in turn folded to form a tandem array of radially arranged loops. These loops are packaged within a fiber that has a final diameter of ~225–250 nm (Rattner and Lin, 1985). A variety of models have been proposed to describe the nature of this last level of chromatin organization. Most of these models view the organization of the fiber as being composed of distinct loops (50–100 kb) which are defined by the attachment of DNA sequences to proteins of the nuclear matrix/scaffold (Benyajati and Worcel, 1976; Rattner and Lin, 1985; Gasser et al., 1986; Belmont et al., 1989). However, the precise role of these matrix/scaffold attachment regions for the compaction of the fiber is still controversial. Nevertheless, the loop organization of chromatin may be important for the regulation of gene expression and replication (Kellum and Schedl, 1991).

Indeed, it appears that chromatin condensation plays a decisive role in the expression of genes. During interphase Eukaryotic chromatin is divided into regions that differ in their degrees of compaction: the transcriptionally active euchromatin and the more compacted heterochromatin (John, 1988). Thus, inactivation of genes can occur when these are brought into the vicinity of heterochromatin leading to posi-

Address all correspondence to Dr. Claudio E. Sunkel, Centro de Citologia Experimental, da Universidade do Porto, Rua do Campo Alegre 823, 4100 Porto, Portugal. Tel: 351 2 69 91 54. Fax: 351 2 69 91 57.

tion effect variegation (PEV)[1]. This phenomenon has been studied extensively in *Drosophila* and more recently in yeast (Aparicio et al., 1991; Paro, 1993; Allshire et al., 1994). There is evidence indicating that PEV is due to the spreading of heterochromatin into adjacent DNA, so that genes placed nearby are inactivated (Demerec, 1940; Hartmann-Goldstein, 1967). The extent of heterochromatinization is variable and consequently the genes are not inactivated in all cells. Modifiers of position effect variegation are chromatin proteins which include the *Drosophila* HP1 (Eissenberg et al., 1990, 1992), the products of the trithorax-group genes (trx-G) (Paro, 1993) and the products of the polycomb-group genes (Pc-G) (Paro and Hogness, 1991; Paro, 1993). The HP1 protein localizes to heterochromatin and might alter gene expression through modification of chromatin structure (James and Elgin, 1986; James et al., 1989). Locke et al. (1988) suggested that factors influencing PEV include heterochromatin proteins that must be assembled from monomeric subunits into a multimeric complex that, in turn, interact with chromatin to form transcriptionally inert heterochromatin.

Besides *Drosophila* HP1, other moderately abundant high mobility group proteins (HMGs) appear to be implicated in the structure of chromatin. Thus, HMG-1 and HMG-2 facilitate bending of DNA (Paull et al., 1993). Furthermore, the *Drosophila* HMG-D, a protein homologue to HMG-1, associates with early embryonic chromatin in the absence of histone H1 (Nen and Travers, 1994). Several lines of evidence are consistent with the possibility that the non-histone chromosomal proteins HMG-14 and HMG-17 are part of a mechanism that confers distinctive properties to chromatin regions containing transcriptionally active genes. These two proteins are the only known non-histones with specific affinity for the nucleosome core particle. It was also shown that the deposition of HMG-17 during replication disrupts nucleosomal organization and the transcriptional potential of chromatin by RNA polymerase III (Crippa et al., 1993).

During mitosis, chromatin assumes a highly condensed organization through the coiling and condensation of the 225–250-nm fiber, thought to involve topoisomerase II (Adachi et al., 1991; Buchenau et al. 1993). The coiled chromatin fiber has been observed both in plant and animal cells during meiosis and mitosis (Rattner and Lin, 1985; Taylor, 1991). It appears that regions where the chromatin fiber fails to undergo coiling result in the appearance of the centromere (primary constriction) and the nucleolus organizer (secondary constriction) in the final chromosomal metaphasic form (Rattner, 1991).

Topoisomerase II is one of the few proteins known to be part of the mitotic chromosome scaffold. The other non-histone proteins identified so far which are associated with the mitotic chromosome localize to centromeric heterochromatin. The centromere is a specialized chromosomal structure that serves a number of functions. It might be required for sister chromatid pairing and cohesiveness and it contains the attachment site for spindle microtubules (kinetochore) which is essential for chromosome movement (Carbon, 1984; Hayden et al., 1990). Most of the centromeric proteins known in human cells were identified through the use of autoimmune CREST sera that reacted against centromere components (Moroi et al., 1980; Earnshaw and Rothfield, 1985). Microinjection of CREST sera into cultured cells during interphase prevents the assembly of a kinetochore, as well as chromosome movement, suggesting that centromere proteins are essential (Bernat et al., 1990, 1991). These include CENP-A (17 kD), CENP-B (80 kD), CENP-C (140 kD), and CENP-D (50 kD). Antibodies raised against purified chromosomal scaffolds identified two other centromere proteins (CENP-E and CENP-F) (Compton et al., 1991; Yen et al., 1991; Rattner et al., 1993). CENP-D is a homologue of RCC1, a protein thought to be required for the establishment or maintenance of proper chromatin structure during interphase. However, its presence at the centromere is presently unclear (Bischoff et al., 1990). The role of CENP-C is also unknown although immunoelectron microscopy indicates that this protein localizes at the inner surface of the kinetochore plate (Saitoh et al., 1992). CENP-B is a DNA-binding protein. It binds a conserved 17-bp sequence (CENP-B-box) present in human centromeric α-satellite DNA. This type of repetitive DNA is present at the centromeres of primates and mouse chromosomes (Muro et al., 1992; Masumoto et al., 1989; Sullivan and Glass, 1991). CENP-B is distributed within the central domain of the centromere that underlies the kinetochore (Cooke et al., 1990). The function of CENP-B is still unclear but it might play a role in the organization of centromeric heterochromatin (Pluta et al., 1992). CENP-A has been purified and shown to be a highly divergent form of histone H3. Unlike the histones, CENP-A is not substituted by protamines during spermatogenesis in mammals (Palmer et al., 1990, 1991). Thus, of all human centromeric proteins identified to date, only CENP-A and CENP-B are likely to play a role in the packaging of chromatin at centromeres (Pluta et al., 1992).

In *Drosophila*, only one centromere-associated protein has been identified. ZW10 associates transiently with centromeres during anaphase and is required for proper chromatid segregation (Williams et al., 1992; Williams and Goldberg, 1994). ZW10 is not found at the centromere of lagging chromosomes present in the mitotic mutants *rod* (Karess and Glover, 1989) and *aar* (Gomes et al., 1993), suggesting that the products of these genes may interact with ZW10.

CENP-B appears to play a role in the packaging of centromeric heterochromatin but has only been identified in primates and mouse. Therefore, the isolation of functional homologues in other species more amenable to genetic analysis would be of great advantage. However, antibodies against CENP-B have consistently failed to identify homologous proteins from other species. Therefore, we have designed a biochemical approach to look for possible functional homologues of CENP-B in *Drosophila*. The work presented here describes the isolation of three polypeptides from *Drosophila* (71, 51, and 35 kD), which display specific CENP-B box-binding activities. Microinjection of these proteins coupled to fluorescein into early embryos indicates that they localize to condensed chromosomes. Immunolabeling of early embryos, using a polyclonal serum raised against all three proteins indicates that they localize preferentially to chromosomal regions known to contain constitutive heterochromatin. During metaphase and anaphase the antigens identified by the serum accumulate near and at the centro-

1. *Abbreviations used in this paper*: HMG, high mobility group protein; Pc-G, polycomb-group genes; PEV, position effect variegation; trx-G, trithorax-group genes.

mere. However, the label does not appear to localize exclusively to heterochromatin since throughout different mitotic stages a faint overall label of chromosome arms is present.

Materials and Methods

Drosophila Strains and Cell Lines

The wild-type strain Oregon R was used throughout this study. For protein purification we used the *Drosophila* Kc cell line and the human cell line Hep-2.

Plasmids and Probes

Plasmid pα(4-1) (Masumoto et al., 1989) contains one (341 bp) human alphoid DNA repeat inserted at the Bam HI pUC19 site. This fragment was used for gel retardation experiments after end-labeling with Klenow. The same fragment was used for footprinting assays. pα(4-1) was digested with Hinc II and Xma I and for the labeling of the L-strand the fragment was end-labeled with Klenow, [α-^{32}P] dCTP, and dGTP. For the labeling of the R-strand the same fragment was labeled with Xma I site with [γ-^{32}P]dATP and T4 polynucleotide kinase. The following oligonucleotide was chemically synthesized (MedProbe, Oslo, Norway) and used as probe or for the construction of the affinity column: CENP-B box oligo: 5'CTAGCTTCG-TTGGAAACGGGA3'/3'GAAGCAACCTTTGCCCTGATC5'. The CENP-B box sequence was designed after Masumoto et al. (1989). The CENP-B box oligonucleotide was used as probe for gel retardation assays by Klenow end-filling of the single-stranded extremities with [α-^{32}P]dCTP or labeled by phosphorylation with T4 Polynucleotide Kinase and [γ-^{32}P]dATP. A concatamer of 210 bp containing 10 copies of the oligonucleotide was inserted at the Xba I site of the pKS+ vector. This concatamer was also used as a probe after end-filling with Klenow.

Nuclear Extracts Preparation

Kc cells were grown in D-22 media (Sigma Chem. Co., St. Louis) supplemented with 5% fetal calf serum (GIBCO BRL, Gaithersburg, MD) at an approximate density of 5×10^6 cells/ml. For one round of purification cells from 1 L culture were collected by centrifugation at low speed in a refrigerated benchtop centrifuge, washed twice with 40 ml of PBS and resuspended in 3–4 ml of buffer A (0.23 M sucrose, 0.01% Triton X-100, 60 mM KCl, 15 mM NaCl, 25 mM $MgCl_2$, 0.5 mM EGTA, 0.15 mM spermidine, 0.15 mM spermine, 14 mM β-mercaptoethanol, 15 mM Tris-HCl, pH 7.4, 2 mM β-glycerophosphate, 2 mM NaF, 0.1 mM PMSF). Cells were broken in a Dounce homogenizer and nuclei were collected by centrifugation through a cushion of 0.4 M sucrose in buffer A at 8,000 rpm for 20 min in a Sorvall RC-5B centrifuge. The pellet, usually 0.5–1.0 ml, was washed with 10 ml of 100 mM NaCl, 100 mM Tris-HCl, pH 7.5, 2 mM β-glycerophosphate, 2 mM NaF, 0.1 mM PMSF and lysed by incubating for 30 min on ice in 10 ml of buffer B (1 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 2 mM β-glycerophosphate, 2 mM NaF, 0.1 mM PMSF). Lysed nuclei were kept frozen at −80°C and thawed at 4°C. Nuclear remnants and DNA were removed by centrifugation at 150,000 *g* for 1 h. Nuclear extracts were dialyzed overnight against BB (10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 2 mM β-glycerophosphate, 2 mM NaF, 0.1 mM PMSF). The precipitate formed during dialysis was removed by centrifugation at 15,000 rpm. Unless specified, all operations were performed at 4°C.

Preparation of the CENP-B Box DNA Affinity Column

500 μg each complementary oligonucleotides were annealed, phosphorylated by T4 Polynucleotide Kinase with ATP and traces of [γ-^{32}P]-ATP, and ligated to form concatamers of 200–1000 bp. The concatamers were then coupled to 10 ml CNBr-activated Sepharose 4B (Pharmacia LKB Nuclear, Gaithersburg, MD) essentially as described by Kadonaga and Tjian (1986). Oligonucleotide binding to the column matrix was monitored by counting the radioactivity present in the first column wash after oligonucleotide coupling.

Protein Purification

Dialyzed nuclear extracts (about 20 ml/1 culture) were loaded onto a DE52 (Whatman Inc., Clifton, NJ) column equilibrated with BB and proteins were eluted with 250 mM KCl and 2 M NaCl in BB. The protein containing fractions were pooled and dialyzed against BB. The CENP-B box-binding activity was monitored by gel retardation assays. The CENP-B box binding fraction (fraction 0.25 M KCl from DE52 column) was chromatographed through a 10 ml CENP-B box-Sepharose column equilibrated in BB plus 0.01% Tween 20. The column was washed with the equilibration buffer and eluted with 250 mM and 1 M KCl in BB plus 0.01% Tween 20. Protein containing fractions were pooled and dialyzed overnight against BB. The affinity column was regenerated by washing with regeneration buffer (2.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at 4°C in storage buffer (0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.02% NaN_3). No proteins were recovered during regeneration of the column. Unless specified all operations were performed at 4°C. Proteins were concentrated in a centricon 100 system (Amicon, Beverly, MA) and stored at −80°C. Protein concentration was determined with a Stratagene Protein Quantitative Assay. The quality of the protein preparation was always assayed by SDS-PAGE followed by silver staining.

Antibodies

Anti-AF1 antibodies were produced by immunizing one rabbit with a mixture of native and denatured AF1 proteins. The preimmune sera did not react with *Drosophila* proteins. The immune serum was cleared from anti-*E. coli* antibodies by chromatography through an *E. coli* proteins-Sepharose column (Pierce Chem. Co., Rockford, IL) and the resulting serum was named Rb5.3. Anti-CENP-B polyclonal antibodies directed against the complete fusion protein expressed in *E. coli* were a gift from Dr. William C. Earnshaw (Johns Hopkins University, Baltimore, MD). Specific antibodies against the individual proteins were immunopurified after blotting to nitrocellulose according to published protocols (Sambrook et al., 1989).

Gel Retardation Assays

Gel retardation assays were performed as described previously (Avides et al., 1990). Binding assays (12.5 μl) typically contained 10 μg of total nuclear extracts or 2 μg DE52 column fractions, or 5–10 ng affinity purified fractions; 0.5–1 ng end-labeled DNA probe; 2.5–10 μg pKS (linearized and supercoiled); in 1× BB. After 15-min incubation at room temperature samples were analyzed in 4% polyacrylamide minigels run in TAE. Gels were dried and exposed to x-ray films (Agfa-Gevaert, Belgium). For competition assays, the amount of pKS was kept constant. For super-shift assays Rb5.3 was included in the binding reactions at a final dilution of 1:100. For the analysis of the protein composition of the complex 5–10 μg of the AF1 proteins or human affinity purified fraction were bound to labeled α-341 DNA. The resulting complexes were separated on a retardation gel and visualized by exposure to x-ray film overnight at 4°C. The complexes were cut out and the slices boiled in 50 μl of SDS-PAGE sample buffer and subsequently applied to a 10% SDS-PAGE gel. Gels were silver stained, dried, and exposed to reveal the location of the DNA probe or used in immunoblot experiments.

Footprint Assays

The DNAse I protection analysis was carried out as described previously (Avides et al., 1990). DNA fragments containing the 341-bp α-satellite DNA repeat were isolated from plasmid pα(4-1) and strand specific end-labeled as described above. The binding reactions (25 μl) contained 1 ng labeled fragment (about 10,000 cpm), 10 ng affinity-purified fractions and were a scale up of the gel retardation assays reactions. After 15-min incubation at room temperature DNAse I (Sigma Chem. Co.) was added at a final concentration of 50 μg/ml and digestion proceeded for 1 min at room temperature. The reaction was terminated by addition of 50 μl STOP solution (20 mM EDTA, 1% SDS, 0.2 M NaCl, 0.25 mg/ml tRNA). After phenol extraction the DNA was ethanol-precipitated, dissolved in 94% formamide, 10 mM EDTA and 0.05% Bromophenol blue, boiled, and loaded into an 8% polyacrylamide sequencing gel. The gel was dried and exposed to x-ray film (Agfa-Gevaert, Belgium).

Immunoblotting

Nitrocellulose membranes (Stratagene Inc., La Jolla, CA) were blocked, incubated with primary antibody, washed, incubated with anti-rabbit IgG conjugated to peroxidase (Vector Labs., Inc., Burlingame, CA) and developed by chemiluminescence using the ECL system (Amersham Corp., Arlington Heights, IL). All procedures were done according to the ECL (Amersham Corp.) protocol for Western blots.

Covalent Coupling of Proteins to Fluorescein

AF1 proteins or histone H1 from calf thymus (Boehringer Mannheim Corp., Indianapolis, IN) were covalently coupled to 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester, utilizing a fluorescein (FLUOS) labeling kit from Boehringer Mannheim. Proteins were concentrated in a centricon 100 device (Amicon) until concentration was ~1 mg/ml. Labeling was controlled by running protein samples in SDS-PAGE gels and observation under UV light.

Microinjection of Drosophila Embryos

Embryos were collected for 30 min, manually dechorionated and injected at 50% egg length according to standard procedures (Santamaria, 1986). They were then incubated in a humid chamber for 20–30 min and fixed with 3.7% formaldehyde as described by Karr and Alberts (1986). After fixation the embryos were washed several times in PBS containing 0.1% Triton X-100 and stained with 0.1 μg/ml Hoechst 33258 or 1 μg/ml propidium iodide in PBS and mounted in 2.5% *n*-porpyl gallate, 85% glycerol. For propidium iodide staining embryos were treated with 2.5 μg/μl RNAse A in PBS for 1 h at room temperature before adding the dye.

Immunostaining of Whole-mount Drosophila Embryos and Neuroblast Preparations

Immunostaining of *Drosophila* embryos was carried out essentially as described by Karr and Alberts (1986). Embryos were incubated with Rb5.3 antibody overnight at 4°C in the presence of 2.5 μg/μl RNAse A at 1:100 dilution. The incubation with fluorescein-coupled anti-rabbit IgG from goat (Vector Labs.) was performed at 4°C for 4 h. DNA was counter stained for 5 min with 1 μg/ml propidium iodide in PBS. Embryos were mounted in 2.5% *n*-propyl gallate, 85% glycerol. Preparations of third instar larval neuroblasts were made as described by Zink and Paro (1993). For immunostaining of squashed neuroblasts the procedures adopted were essentially the same, except that incubations with antibodies were performed for 1 h at room temperature.

Microscopy and Image Analysis

A confocal laser scanning microscope MRC-600 was used for collecting digital images of optical sections through fixed embryos or squashed neuroblasts. Image analysis was performed using the COMOS software (Bio Rad Labs.).

Results

Nuclear Protein Extracts from Drosophila Kc Cells Exhibit a Binding Activity Towards a Human Centromeric DNA Sequence

Nuclear extracts from *Drosophila* Kc cells were tested by gel retardation for the presence of a specific binding activity towards the human α-341 centromere DNA sequence. The result of one of those experiments is shown in Fig. 1 *A*. Kc cells nuclear extracts were incubated with radiolabeled α-341 DNA and the resulting protein/DNA complex can be seen on lane *2*. This complex is no longer detected after addition of a 10-fold molar excess of unlabeled α-341 DNA to the binding reactions (lane *4*), indicating that the protein mixture contains a specific α-341–binding activity. This activity seems to result from binding to the CENP-B box sequence. Addition of a 10-fold molar excess of a 21-mer oligonucleotide containing the 17 bp CENP-B box prevents the formation of the radioactive complex (lane *6*). As a positive control, the same α-341 DNA-binding assays were performed using human nuclear extracts from Hep-2 cells. The complex formed in this binding reaction is shown on lane *3*. This complex seems to result from specific interactions between proteins of the mixture and the CENP-B box sequence. The binding is effectively competed by unlabeled α-341 DNA (lane *5*) or the CENP-B box oligonucleotide (lane *7*).

To ensure that the Kc cells nuclear extracts contained a specific CENP-B box binding activity, we used the CENP-B box oligonucleotide as a probe in retardation assays. The results are shown in Fig. 1 *B*. The complex formed between *Drosophila* or human nuclear proteins and the CENP-B box oligonucleotide is shown in lanes *2* and *3* respectively. Both of these complexes are no longer detected after addition of a 10-fold molar excess of either unlabeled oligonucleotide or

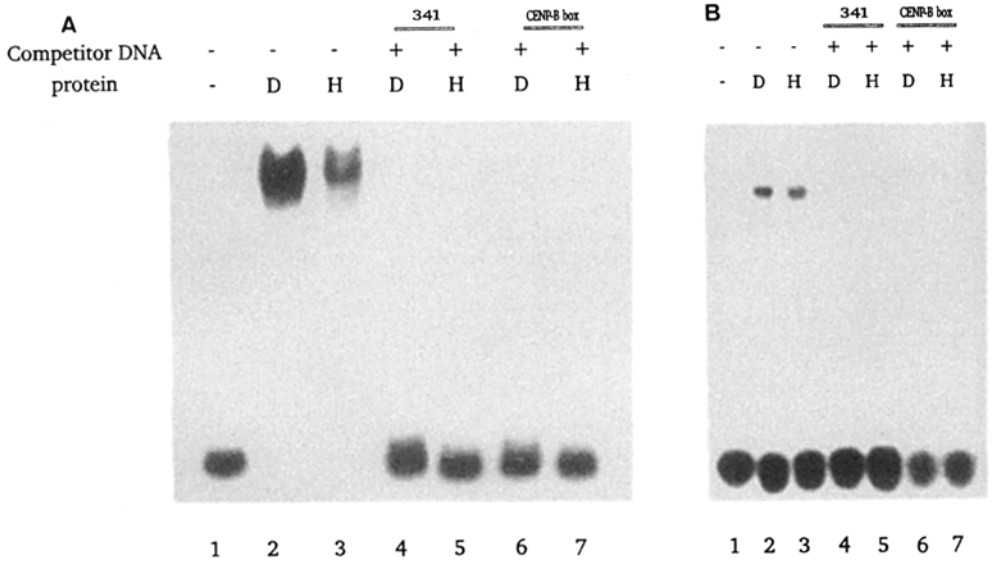

Figure 1. Alphoid DNA and CENP-B box-binding activities in *Drosophila* and human cells nuclear extracts assessed by gel retardation. (*A*) Alphoid DNA-binding activity. Reactions included no proteins (lane *1*), *Drosophila* nuclear extracts (lanes *2*, *4*, and *6*) or human nuclear extracts (lanes *3*, *5*, and *7*). A protein/α-341 DNA complex is visible on lanes *2* and *3*, and is competed by a 10-fold molar excess of unlabeled α-341 (lanes *4* and *5*) or CENP-B box oligonucleotide (lanes *6* and *7*). (*B*) CENP-B box DNA-binding activity. This DNA mobility shift assay is similar to the one described for Fig. 1 *A*, except that the CENP-B box oligonucleotide was used as labeled DNA.

α-341 DNA, indicating that they are the result of specific interactions.

Purification of the CENP-B Box Binding Activity from Drosophila Kc Cells

In order to purify the CENP-B box binding activity from Kc cells, nuclear protein extracts from *Drosophila* Kc cells were fractionated on a DE52 ion-exchange column. This column was washed with a buffer containing 60 mM KCl and eluted with 250 mM KCl and 2 M NaCl. The CENP-B box-binding activity of the eluted fractions was monitored by gel retardation assays using a concatamer of the CENP-B box oligonucleotide (Fig. 2 *A*). This activity was found to elute at 250 mM NaCl (lane *4*) and it was further purified by affinity chromatography on a CENP-B box-Sepharose column. The column was washed with 60 mM KCl and eluted with 250 mM and 1M KCl. The CENP-B box-binding activity was retained by the column, eluting at 1 M KCl (lane *9*). Some of this activity is also observed in the column flow-through (lane *6*). This is probably due to overloading of the column. The protein composition of the fractions was analyzed by SDS-PAGE (Fig. 2 *B*). After silver staining the affinity-purified CENP-B box-binding fraction (AF1) was shown to consist of three predominant polypeptides with apparent molecular mass of 71, 50, and 31 kD (lane *7*). The whole purification procedure was applied to human nuclear protein extracts from Hep-2 cells and the resulting 1 M KCl fraction from the affinity column was found to consist of three major polypeptides. One of which had an apparent molecular mass of 80 kD on SDS-PAGE and was subsequently identified as CENP-B by immunoblot (Fig. 3). Table I contains a summary of the purification of the AF1 proteins.

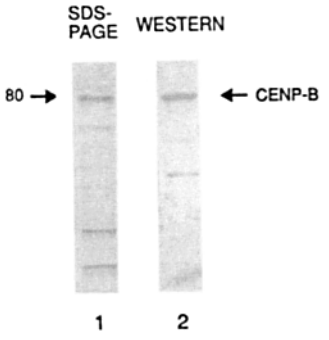

Figure 3. Purification of human CENP-B from Hep-2 cells. SDS-PAGE analysis of 2 μg of the final purified fraction as shown by silver staining (lane *1*). Western blot assay of the purified fraction with anti-CENP-B polyclonal antibody diluted 1:500 (lane *2*).

AF1 Proteins Recognize the CENP-B Box Sequence on α-341 DNA

The previous gel retardation experiments suggested that the purified proteins bind specifically to the CENP-B box sequences. However, in order to ensure this specificity, DNAse I protection experiments were performed using α-341 DNA as a probe (Fig. 4 *A* and *B*). The results show that both strands of the CENP-B box sequence are protected by the AF1 proteins after digestion with DNAse I. This suggests that the AF1 proteins bind in vitro to the CENP-B box on the α-341 DNA. A similar pattern of protection to DNAse I digestion is obtained with human CENP-B–enriched fraction prepared by us. Minor differences are present in the protection pattern originated by human as compared to the *Drosophila* proteins. *Drosophila* proteins protect a few more base pairs from DNAse I digestion than human proteins. We do not know if such differences arise from differential contacts with the helix or if they are due to protein steric hindrance to cleavage by DNAse I.

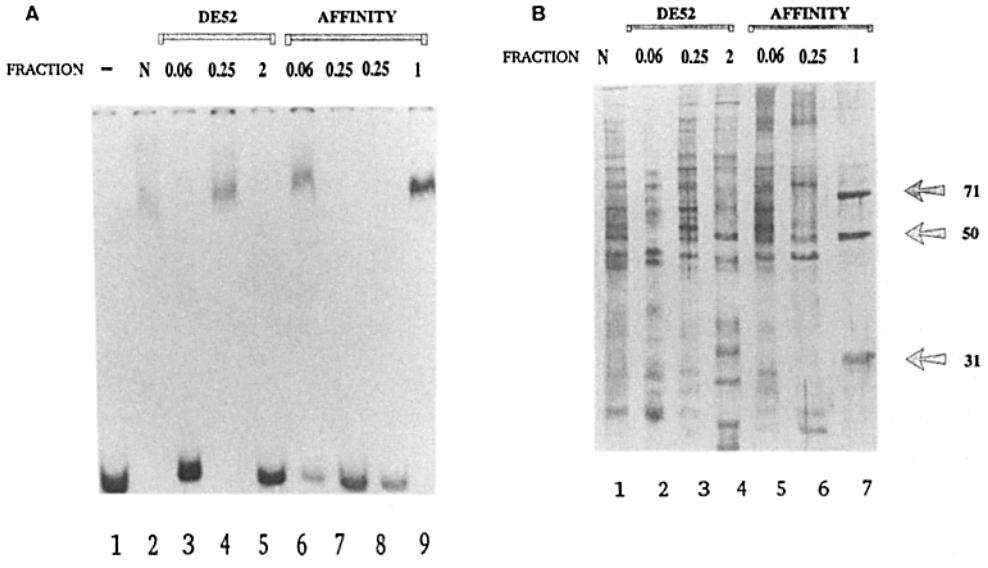

Figure 2. Purification of the CENP-B box-binding activity from *Drosophila* Kc cells by DE52 and oligonucleotide-Sepharose chromatography. (*A*) The binding activity of the column fractions was assessed by gel retardation assays using a concatamer of the CENP-B box oligonucleotide as a probe. No protein control (lane *1*). Binding activity observed when total nuclear extracts (*N*) from Kc cells are used (lane *2*). No binding activity was detected in the DE52 flow-through (lane *3*). However, most of the binding activity elutes at 0.25 M NaCl (lane *4*) and nothing remains after elution with 2 M NaCl (lane *5*). The flow-through from the affinity column still has DNA-binding activity which is probably due to overloading (lane *6*). No binding activity is observed after elution at 0.25 M KCl (lanes *7* and *8*; lane *8* has twice as much protein as lane *7*). Binding activity is retained by the CENP-B box column eluting 1 M KCl (lane *9*). (*B*) Protein composition of the various column fractions was analyzed by SDS-PAGE followed by silver staining. 5 μg of each protein sample was applied in lanes *1* to *6*. Nuclear proteins from Kc cells (lane *1*), 0.06 M KCl eluate from DE52 (lane *2*), 0.25 M KCl eluate from DE52 (lane *3*), 2 M NaCl eluate from DE52 (lane *4*), 0.06 M KCl from affinity column (lane *5*) and 0.25 M KCl eluate from affinity column (lane *6*). 2 μg of the 1 M KCl eluate from the affinity column were applied to lane *7*.

Table I. Purification of CENP-B Box-binding Polypeptides

Fraction	Volume	Total Protein	DNA Binding Activity*		Yield		Purification	
			Total (U)	Specific (U/μg)	Per Step	Overall	Per Step	Overall
	ml	*μg*	*U*	*U/μg*	*%*		*fold*	
Nuclear extract	65	91,000	34,000	0.37	–	–	1	1
DE52	25	19,600	23,800	1.2	70	70	3.2	3.2
Oligonucleotide-Sepharose	2	1	4,760	4,760	20	14	4,000	13,000

* One unit corresponds to the quantity of protein necessary to bind 1 fmol of alphoid DNA.

Anti-AF1 Polyclonal Antibody Recognizes a Similar Set of Polypeptides in Several Drosophila Protein Extracts

In order to characterize further the AF1 fraction, polyclonal antibodies were made against the AF1 proteins in rabbit. The results obtained with the immune serum (Rb5.3) are shown in Fig. 5 *A*. It recognizes a similar set of polypeptides in total and nuclear Kc cells extracts, early embryonic extracts and in extracts from third instar brains. All extracts show a constant band of 71 kD. However, the 50-kD band appears as a doublet or a triplet depending upon the origin of the extract used. Affinity-purified extracts have a single 50-kD band while extracts from Kc cells, embryos and third instar larval brains extracts contain two or three bands. Phosphatase treatment of these extracts before electrophoresis does not alter the patterns of the 50-kD antigen in the different extracts (data not shown). Rb5.3 also recognizes the 31-kD polypeptide in the AF1 fraction (Fig. 5 *B*) but the reaction in whole extracts is very weak and only a faint band is seen. This is probably due to a very low abundance of this polypeptide.

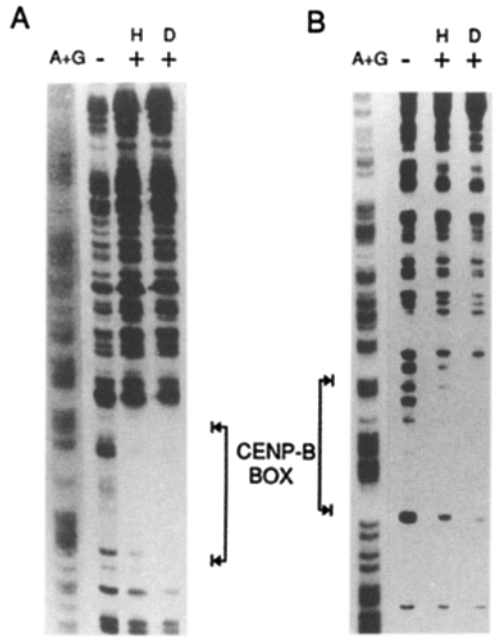

Figure 4. DNAse I footprinting of purified AF1 and CENP-B on α-341 DNA. A+G-sequencing reactions and DNAse I digestion of the L-strand (*A*) and R-strand (*B*) are shown. 1 ng of end-labeled DNA at one of the two complementary strands was incubated without (−) or with either 5 ng of the AF1 proteins (*D*) or 5 ng of the affinity-purified CENP-B (*H*) before DNAseI digestion.

If the gel is overloaded the 31-kD polypeptide is detected by Rb5.3 antibodies as shown in Fig. 5 *C*. We were not able to detect any polypeptide recognized by Rb5.3 in nuclear extracts from human Hep-2 cells (Fig. 5 *A*, lane *3*). In addition, specific antibodies immunopurified against individual polypeptides from the AF1 fraction only recognize the protein band used for immunopurification (Fig. 5 *D*). These results clearly suggest that the lower molecular mass bands are not due to partial degradation of the high molecular mass band.

Rb5.3 Is Able to Bind AF1 in Solution and Bound to DNA

We next intended to test if the polyclonal antibodies produced were able to recognize the native AF1 polypeptides in solution either free or bound to DNA. The results of supershift assays are shown in Fig. 6. When antibodies are incubated with labeled DNA (CENP-B box concatamer) in the absence of other proteins no complex is detected (lane *1*) showing that under the conditions employed Rb5.3 antibodies do not bind to DNA. If antibodies are incubated with the AF1 proteins before the addition of labeled DNA (lane 2) or if antibodies are incubated with DNA before the addition of protein, then a complex is formed. The mobility of this complex is slower than the mobility of the AF1/CENP-B box complex originated in the absence of antibodies (lane *4*). This suggests that the low mobility complex is composed of antibodies bound to the protein/DNA complex. This type of assays led us to conclude the Rb5.3 recognises the native proteins in solution and that the bound antibodies do not impair the ability of proteins to associate with DNA. It is therefore likely that the epitopes recognised by Rb5.3 are outside the protein DNA binding domains.

All Three AF1 Proteins Are Present in the Complex with the α-341 DNA

The protein composition of the complex found after gel retardation was determined using immunopurified antibodies against each individual protein of the AF1 faction. For this experiment, the α-341 DNA fragment was used as probe and the complex transferred to nitrocellulose by electroblotting. The results are shown in Fig. 7 *A*. Four lanes with equal amount of DNA and protein were run in parallel. After blotting, the nitrocellulose was cut into four stripes and probed either with the whole serum (lane *1*) or with immunopurified antibodies against the 71-kD protein (lane 2), the 50-kD protein (lane *3*), and the 31-kD protein (lane *4*). The results clearly show that the complex formed between the AF1 proteins and the α-341 DNA contains all three proteins. Further confirmation of these results was obtained by analyzing the

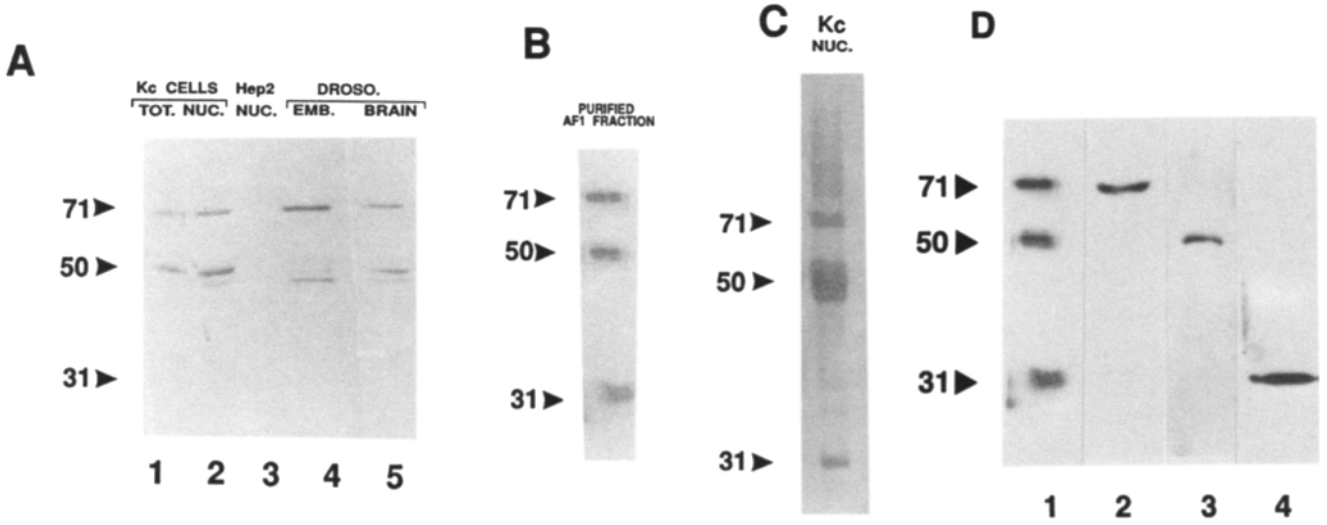

Figure 5. Western blot analysis of the AF1 antigens. (*A*) Immunoblot of the AF1 antigens in different *Drosophila* cell types. 20 μg of Kc total cell extracts (lane *1*), 10 μg of nuclear extracts from Kc cells (lane *2*), 10 μg of human Hep2 cells nuclear extracts (lane *3*), 10 μg of early embryonic extract (lane *4*), and 10 μg of total brain extracts (lane *5*). (*B*) Analysis of the reactivity f Rb5.3 against the AF1 proteins by Western blot. 2 μg of protein were loaded onto the gel. (*C*) Visualization of the 31-kD polypeptide in nuclear extracts from Kc cells. The gel was overloaded with 50 μg of the extract to allow visualization of the lower molecular mass band. The Rb5.3 serum was used at 1:500 dilution. (*D*) Western blot analysis of immunopurified antibodies against each of the individual polypeptides from the AF1 fraction. 2 μg of AF1 proteins were loaded in each lane and all immunopurified antibodies were used at a final concentration of 35 μg/ml. Anti-71-kD protein (lane *1*), anti-50-kD protein (lane *2*), and anti-31-kD protein (lane *3*).

proteins recovered from preparative gel retardation assays (Fig. 7 *B*). For this experiment, duplicate gel retardations containing either the AF1 proteins (lanes *1*, *3*, and *5*) or human affinity purified proteins (lanes *2*, *4*, and *6*) were run. Complexes were detected by overnight autoradiography and the gel fragment was removed, casted into SDS-PAGE gel, and separated. One set was silver stained in order to visualize the proteins (lanes *1* and *2*). Since DNA is also stained by this method, the same gel was dried and exposed overnight to determine the location of the free DNA probe (lanes *3* and *4*). They both show at the top the free DNA probe and in lane *1* the three AF1 proteins can be clearly observed. The human complex contains a single 80-kD band (lane *2*). The identity of the three *Drosophila*, as well as that of the human protein was determined by immunoblotting. The results show that serum Rb5.3 stains all three AF1 proteins clearly indicating that they were present in the original gel retardation complex (lane *5*). Human anti-CENP-B antibodies were used to identify the 80-kD protein present in lane *2* as CENP-B (lane *6*).

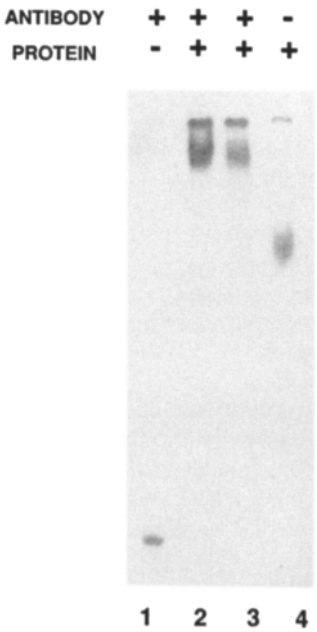

Figure 6. Super-shift analysis of the AF1/CENP-B box complexes. Control assay for the ability of the Rb5.3 serum to bind the probe on its own (lane *1*). Proteins were incubated with the probe before the antibody was added (lane *2*). Proteins were incubated with the antibody after the addition of the probe (lane *3*). Control assay to show the formation of the complex in the absence of antibodies. All reactions contained 0.1 ng of labeled DNA. 10 ng of the AF1 proteins were used and the antibody was diluted 1:100.

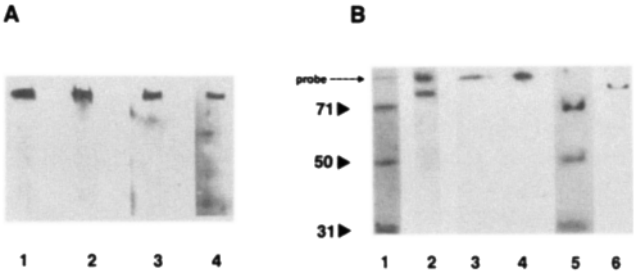

Figure 7. Analysis of the protein composition of the AF1/α-341 DNA complex. (*A*) Gel retardation gels were blotted to nitrocellulose and probed with serum Rb5.3 (lane *1*) or immunopurified antibodies against the 71-kD protein (lane *2*), the 50-kD protein (lane *3*), and the 31-kD protein (lane *4*). (*B*) Preparative gel retardation assays. The protein composition of the *Drosophila* or the human complex were analyzed by SDS-PAGE and silver stain (lanes *1* and *2*). The same gel was exposed overnight to reveal the location of the free probe (lanes *3* and *4*). Parallel reactions were prepared and separated as before except that they were not stained but blotted to nitrocellulose and probed with either serum Rb5.3 (lane *5*) or anti-CENP-B antibodies (lane *6*). All antibodies were used at 1:500 dilution.

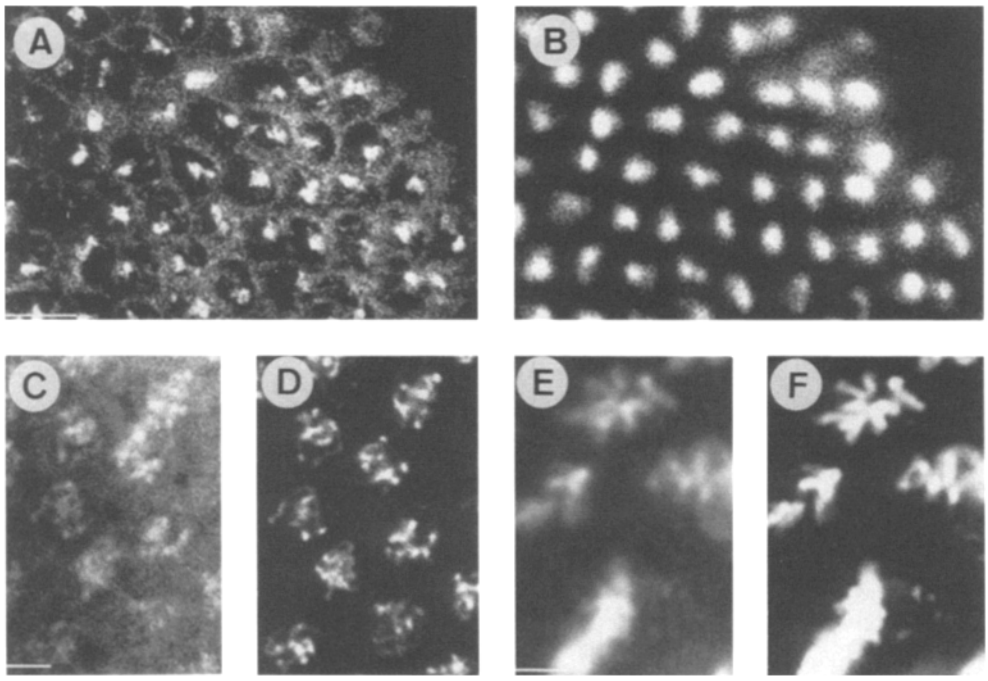

Figure 8. Microinjection of labeled histone H1 or AF1 proteins into early *Drosophila* embryos. The preparations were observed by confocal laser microscopy and the images constructed from an average of 15 optical sections. AF1 proteins were labeled with FITC (*C* and *E*) and the DNA counter stained with propidium iodide (*B*, *D* and *F*). Low magnification of an embryo injected with labeled histone H1 (*A* and *B*). High magnification of different preparations showing either prometaphase nuclei (*C* and *D*) or metaphase nuclei (*E* and *F*). Bars: (*A* and *B*) 20 μm; (*C* and *D*) 5 μm.

Localization of the AF1 Proteins in Drosophila Embryos by Microinjection of the Fluorescein-labeled Polypeptides

In order to determine the localization of the AF1 polypeptides, the whole protein fraction was fluorescently labeled by covalent coupling to fluorescein followed by microinjection into early *Drosophila* embryos. The embryos were injected within the first hour of embryogenesis and then allowed to continue development for several periods of time. They were then fixed and examined by confocal laser microscopy. Examples of the results obtained by microinjection are shown on Fig. 8. As a positive control histone H1 from calf thymus was coupled to fluorescein and microinjected into the embryos (Fig. 8, *A* and *B*). The results show that this protein colocalizes with the chromatin. BSA was used as a negative control and it showed no specific localization within the embryo (data not shown). Embryos were then injected with the AF1 fraction tagged with fluorescein. The results shown in Fig. 8, *C* and *E* indicate that the AF1 proteins localize to the condensed chromatin. The chromosomes appear stained throughout, although some regions are stained with more intensity. Some of these regions are known to contain constitutive heterochromatin.

AF1 Localization by Indirect Immunofluorescence

The polyclonal serum Rb5.3 was used to determine the localization of the AF1 proteins during mitosis in early *Drosophila* embryos. The results are shown in Fig. 9. During interphase we observed a speckled pattern of staining throughout the chromatin. These specks seem to converge into a few large dots per nucleus by prometaphase. During metaphase the proteins appear concentrated in the middle of metaphase plates in zones encompassing the centromeres of all chromosomes. At early anaphase the proteins localized mostly near the centromeres of chromatids. During late anaphase the Rb5.3 stains an area of the chromatids which is nearest to the poles, although it becomes more diffuse. At telophase the staining pattern concentrates into a single large dot within individual nuclei.

The immunolocalization of the AF1 proteins in embryos, although indicative of the distribution pattern of these proteins during mitosis, does not provide much detailed information about the exact chromosomal localization of these proteins. To address this question, mitotic chromosome spreads from third instar larval brains were also stained with antibody Rb5.3. Fig. 10 shows that AF1 proteins are concentrated in the pericentromeric regions of the autosomals, although faint chromosomal arm staining is also observed. The patterns of staining observed amongst different preparations did not vary significantly from those shown in Fig. 10.

Discussion

The AF1 Proteins Associate Specifically with the Human CENP-B Box Sequence

In this report we present the purification of three polypeptides by their ability to bind to the human centromeric DNA sequence CENP-box. As determined by gel retardation assays and footprinting experiments, these polypeptides exhibit a specific binding activity towards the sequence CENP-B box. The values obtained for the specific binding activity throughout the purification procedure were similar to those published previously for the purification of CENP-B (Muro et al., 1992). They reported the purification of CENP-B from HeLa cells, through a two-step procedure including an ion-exchange chromatography and oligonucleotide affinity chromatography. These purified fractions included a major polypeptide of apparent mobility on SDS-PAGE gels of 80 kD that was identified as CENP-B, as well as two minor polypeptides comigrating as a 65-kD doublet, one of which might be the result of proteolytical degradation of CENP-B. The other band was not recognized by polyclonal anti-

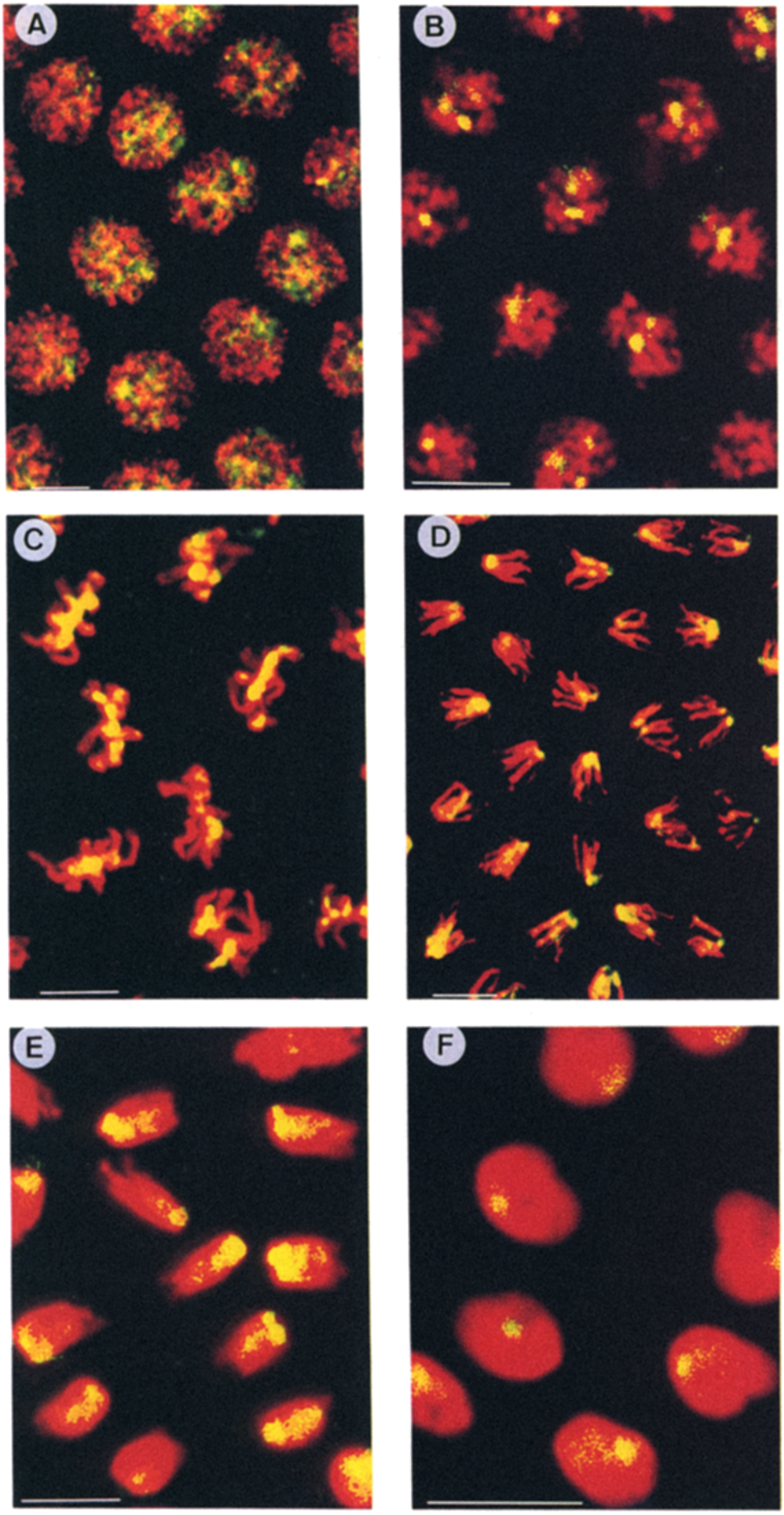

Figure 9. Immunolocalization of AF1 proteins during mitosis in early *Drosophila* embryos. Preparations were observed under a confocal laser microscope using the same conditions described before. DNA staining is shown in red and antibody in green. Colocalization of the two staining patterns will appear as yellow. The different images were obtained from different embryos at different mitotic stages. (*A*) Interphase; (*B*) prometaphase; (*C*) metaphase; (*D*) anaphase; (*E*) late anaphase-early telophase; and (*F*) telophase. The polyclonal serum Rb5.3 was used at 1:50 dilution. Bar, 5 μm.

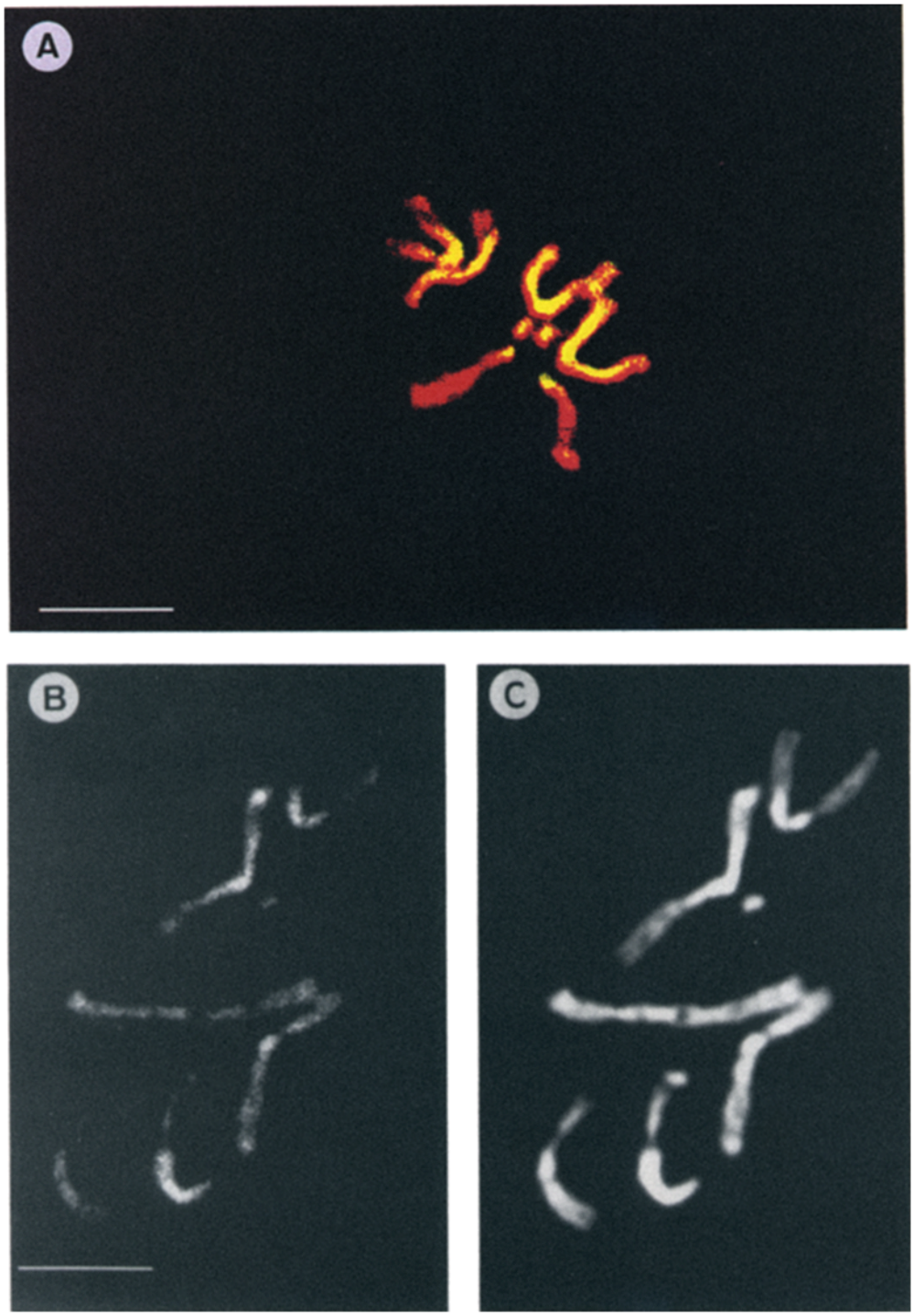

Figure 10. Immunolocalization of AF1 proteins in neuroblast squashes analyzed by confocal laser microscopy. (*A*) Merged image of DNA (red) and antibody staining (green). Unmerged images of a different neuroblasts preparation to visualize Rb5.3 staining in *B* and DNA staining in *C*. Individual chromatids are not yet visible in this preparation. The pattern of staining of Rb5.3 shows significant accumulation within heterochromatic regions of the autosomals. In the fourth and X chromosome the distribution of Rb5.3 staining appears to be more diffuse. The Rb5.3 serum was used at 1:50 dilution. Bar, 5 μm.

CENP-B antibodies and it is possible that this polypeptide is not derived from CENP-B. We used a similar protocol for purification of *Drosophila* CENP-B box binding polypeptides. The purified fraction we obtained consisted of three polypeptides of apparent molecular mass 71, 50, and 31 kD. Our protocol was tested for the ability to purify human CENP-B from Hep-2 cells. We obtained a fraction consisting of an 80-kD polypeptide identified as CENP-B by Western blot analysis together with 65- and 28-kD polypeptides that do not react with anti–CENP-B polyclonal sera. This fraction binds α-341 DNA in gel retardation assays recognizing the sequence CENP-B box on DNAse I protection experiments. Muro et al. (1992) reported that CENP-B was unable to bind an oligo containing only the 17-bp CENP-B box, suggesting that the adjacent sequences were also important for the formation of the complex. We used concatamers of a 21-mer oligonucleotide containing the CENP-B box sequence both for the construction of the DNA affinity column and gel retardation assays. The larger DNA fragments used in these experiments may explain the CENP-B–binding activity that we observed.

AF1 proteins, like CENP-B, are able to specifically bind

CENP-B box sequences on gel retardation assays. DNAse I protection experiments also show that the AF1 proteins give rise to a protection pattern similar to the one produced by CENP-B as was established by Moroi et al. (1992). The only difference is that the AF1 footprint seems to extend a few more nucleotides than that observed when CENP-B is used. The differences in the two footprinting patterns may arise from contacts in the DNA on different bases or from a different size or conformation of the protein complexes. CENP-B has been shown to bind the CENP-B box as a dimmer (Yoda et al., 1992). However, the AF1/DNA complex contains all three polypeptides which might induce the formation of a larger complex. This could render DNAse I access to some bases impossible.

Characterization of the AF1 Antigens

In order to characterize further the AF1 antigens we prepared a polyclonal serum against all three proteins. Western blots of *Drosophila* nuclear extracts indicate that these three proteins are not originated by proteolysis of a larger precursor. This conclusion is supported by the observation that the three proteins are always observed in different extracts prepared at different times and the addition of several protease inhibitors does not alter this pattern. In addition, antibodies immunopurified against individual polypeptides only recognize the original protein in total extracts or AF1 fraction.

The protocol used to purify the AF1 proteins from *Drosophila*, also works for the isolation of the human CENP-B. However, the Rb5.3 antibody was not able to recognize any protein from either total or affinity purified human cell extracts. These results would suggest that the AF1 proteins share with CENP-B the ability to bind the CENP-B box but do not share significant sequence identity. This is not unexpected since anti-CENP-B antibodies (Earnshaw, W. C., unpublished results) or other Human ACA sera (Machado and Sunkel, unpublished results) have consistently failed to recognize any *Drosophila* centromeric associated polypeptide.

The 50-kD antigen from the AF1 fraction appears to show heterogeneity in nuclear extracts from different cell types as shown in Western blot experiments. This could be due to the presence of a family of related polypeptides or reflect posttranslational modifications of a single polypeptide. However, only one of these forms is found after affinity purification. This could either be due to loss of posttranslational modifications during extraction or could indicate that only one of the forms participates in the formation of the protein/DNA complex. Since all affinity purifications give only a single 50-kD polypeptide, it is likely that only one form participates in the formation of the protein/DNA complex. Furthermore, phosphatase treatment of the extracts before electrophoresis does not alter this pattern, indicating that the heterogeneity observed is probably not due to differential phosphorylation.

The Rb5.3 serum was also used to determine whether any of the three antigens participates in the formation of the complex and whether the DNA binding affinity could be altered by the antibodies. Super-shift assays indicate that at least one of these proteins is a component of the protein/DNA complex observed on gel retardation assays. The results also show that incubation of the AF1 proteins with the Rb5.3 serum before DNA binding does not alter their ability to form the complex. The composition of the complex was further characterized by blotting to nitrocellulose and then probing a blot with immunopurified antibodies against each protein present in the AF1 fraction. The results clearly show that all three proteins are present in the complex with the α-341 DNA. These results were further confirmed by preparative gel retardation assays, followed by SDS-PAGE of the complex. After separation, the gel was stained and all three proteins can be observed. A parallel lane was blotted to nitrocellulose and probed with serum Rb5.3 which stained the three polypeptides. The complex does not appear to form by sequential incorporation of the three proteins. Gel retardation experiments using the α-341 DNA as probe, and increasing amounts of the AF1 fraction, shows the formation of a single complex. There were no intermediate complexes observed with low concentrations of the AF1 fraction (Avides and Sunkle, unpublished results). The results presented above clearly indicate that all three proteins are present in the complex, however, we have been unable to determine which of them actually binds the DNA. As a positive control, the same experiment was repeated with the human extracts and CENP-B was identified as the main component of the complex.

The AF1 Proteins Can Be Incorporated into Live Nuclei

Microinjection of the AF1 protein fraction was used to determine whether these polypeptides were still functional in vivo and also to ascertain their general localization within the developing embryo. The results show that one or more of the AF1 proteins can be incorporated into the chromatin of live embryos without any significant alterations of the nuclear multiplication pattern. Most embryos do not show mitotic abnormalities and they contain a large number of nuclei. Incorporation of the fluorescein-AF1 proteins was relatively low and could only be seen in embryos that had reached cycle 9, after migration of the nuclei to the cortex. Detailed analysis of the protein fluorescence pattern throughout mitosis reveals that it is identical to the staining pattern of DNA produced by Hoechst 33258 and propidium iodide. As these dies bind preferentially to heterochromatin (John, 1988) we concluded that AF1 proteins concentrated in heterochromatic regions.

The AF1 Proteins Localize to Chromatin During All Stages of Mitosis

The immunolocalization of the AF1 proteins during the different stages of mitosis was determined using the polyclonal serum Rb5.3 using fixed early *Drosophila* embryos. The results show that these antigens associate to chromatin throughout the nuclear mitotic cycle. During interphase they show a speckled distribution within the chromatin with one or two larger areas of more concentrated staining. At prophase the staining appears highly localized to a few areas of the chromatin and at metaphase it concentrates mostly within the center of the metaphase plate. During anaphase most of the staining remains associated to the chromatid regions closest to the poles and therefore near or at the centromeres. The staining is similar during late anaphase, though the area stained becomes more diffuse. At telophase the antibody staining is confined to a single area. Both, the metaphase and anaphase staining patterns, suggest that the

AF1 proteins accumulate at or near the centromeres. Essentially the same results were obtained after microinjection of labeled AF1 proteins. Although no chromocenter is observed in *Drosophila* embryos (Huettner, 1933), it is tempting to speculate that the AF1 proteins might be localized to a heterochromatic rich region during interphase. However, during mitosis these proteins associate mainly to constitutive heterochromatin. Nevertheless, low level of staining of chromatids is also observed during metaphase and anaphase. At least two hypotheses can be put forward to explain this result. This pattern of staining could be due either to the differential localization of one or two of the three AF1 antigens, in which one of them accumulates primarily to the heterochromatin. Alternatively, it could represent differential localization of all three polypeptides. However, we have been unable to determine the localization of each of the three AF1 antigens and therefore we cannot distinguish among these two possibilities. The pattern of localization of the AF1 proteins is clearly different from that of CENP-B which is always associated with chromatin as discrete dots clearly confined to the centromeres of most human chromosomes (Pluta et al., 1992).

Immunolocalization of the AF1 Protein in Chromosome Spreads

In order to further localize the AF1 proteins within individual chromosomes we used the antibody Rb5.3 directly onto third instar larval neuroblasts spreads which had not been treated with colchicine. The results indicated that the AF1 proteins appear to localize preferentially to sites of constitutive heterochromatin. However, the interpretation of these results is complicated by the fact that the fixation/stretching protocol utilized may solubilize some of the antigens. In some cases the centromeric accumulation of the antigens is very clear, although the arms of chromosomes are also stained. Therefore, the question remains as to why proteins that exhibit such specificity of binding towards CENP-B box show such a widespread distribution. Furthermore, we do not know if the *Drosophila* genome contains any CENP-B box related sequences within centromeric heterochromatin. However, results from our laboratory suggest that *Drosophila* centromeric DNA has sequences related to the CENP-B box, although no perfect match has been identified so far (Coelho and Sunkel, unpublished observations). Maybe the specificity of the AF1 proteins towards the CENP-B box sequence is only observed in the context of α-341 DNA.

The occurrence of the CENP-B box sequence varies among human chromosomes and no CENP-B box sequence was found at the Y chromosome (Choo et al., 1991). As Pluta et al. (1992) point out the most reasonable solution to the paradox is that CENP-B is functionally redundant and that another chromosomal component is able to perform the same role. The function of this component might not only be restricted to the assembly of a functional centromere containing a kinetochore at its external surface. It may also be required for the organization of centromeric and pericentromeric heterochromatin. Given the distribution of the AF1 antigens it is conceivable that at least one of the three proteins we have purified could correspond to a functional homologue of CENP-B. The cloning of the genes which encode the three proteins is now in progress. This will allow us to begin a more detailed characterization of the antigens and to determine their individual localization within the mitotic chromosome. Furthermore, the localization of these genes within the genome of *Drosophila* will allow their genetic analysis which will open the way to study their function.

The authors are grateful to Dr. Tunedo Okazaki for providing plasmid pα(4-1), and to Dr. William C. Earnshaw for giving us the anti-CENP-B polyclonal antibody. We would also like to thank Paula Coelho for her help during the initial stages of this project; Paula Sampaio for the protein extracts from *Drosophila* brains; and Inês Chaves and Cristina Machado for protein extracts from early embryos. We also thank Elsa Bronze-da-Rocha, José Catita, Jorge Pedrosa, and Joana Perdigão for their help during preparation of the Rb5.3 serum and Álvaro Tavares for helping in the preparation of the figures and for his useful comments on the manuscript.

M. C. Avides was supported by a scholarship from Junta Nacional De Investigaçao Científica e Tecnológica (JNICT). This work was supported by grants to C. E. Sunkel from JNICT.

Received for publication 9 June 1994 and in revised form 29August 1994.

References

Adachi, Y., M. Luke, and U. K. Laemmli. 1991. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell.* 64:137–148.

Allshire, R. C., J.-P. Javerzat, N. J. Redhead, and G. Cranston. 1994. Position effect variegation at fission yeast centromeres. *Cell.* 76:157–169.

Aparicio, O. M., B. L. Billington, and D. E. Gottschling. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell.* 66:1279–1287.

Avides, M. C., C. E. Sunkel, P. Moradas-Ferreira, and C. Rodrigues-Pousada. 1990. Properties and partial characterization of the heat shock factor from Tetrahymena pyriformis. *Eur. J. Biochem.* 194:331–336.

Belmont, A. S., M. B. Braunfeld, J. W. Sedat, and D. A. Agard. 1989. Large-scale structural chromatin domains with mitotic and interphase chromosomes in vivo and in vitro. *Chromosoma (Berl.).* 98:129–143.

Benyajati, C., and A. Worcel. 1976. Isolation, characterization, and structure of the folded interphase genome of Drosophila melanogaster. *Cell.* 9: 393–407.

Bernat, R. L., G. G. Borisy, N. F. Rothfield, and W. C. Earnshaw. 1990. Injection of anticentromere antibodies in interphase disrupts events required for chromosome movement in mitosis. *J. Cell Biol.* 111:1519–1533.

Bernat, R. L., M. R. Delannay, N. F. Rothfield, and W. C. Earnshaw. 1991. Disruption of centromere assembly during interphase inhibits kinetochore morphogenesis and function in mitosis. *Cell.* 66:1229–1238.

Bischoff, F. R., G. Maier, G. Tilz, and H. Ponstingl. 1990. A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by rcc1, a gene implicated in the onset of chromosome replication. *Proc. Natl. Acad. Sci. USA.* 87:8617–8621.

Buchenau, P., H. Saumweber, and D. Arndt-Jovin. 1993. Consequences of topoisomerase II inhibition in early embryogenesis of Drosophila revealed by in vivo confocal laser scanning microscopy. *J. Cell Sci.* 104:1175–1185.

Carbon, J. 1984. Yeast centromeres: structure and function. *Cell.* 37:351–353.

Choo, K. H., B. Vissel, A. Nagy, E. Earle, and P. Kalitsis. 1991. A survey of the genomic distribution of alpha satellite DNA on all human chromosomes and derivation of a new consensus sequence. *Nucleic Acid Res.* 19:1179–1182.

Compton, D. A., T. J. Yen, and D. W. Cleveland. 1991. Identification of novel centromere/kinetochore-associated proteins using monoclonal antibodies generated against human mitotic chromosome scaffolds. *J. Cell Biol.* 112:1083–1097.

Cooke, A., R. L. Bernat, and W. C. Earnshaw. 1990. CENP-B: a major human centromere protein located beneath the kinetochore. *J. Cell Biol.* 100: 1475–1487.

Crippa, M. P., L. Treischmann, P. J. Alfonso, A. P. Wolffe, and M. Bustin. 1993. Deposition of chromosomal protein HMG-17 during replication affects the nucleosomal ladder and transcriptional potential of nascent chromatin. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3855–3864.

Demerec, M. 1940. Genetic behaviour of euchromatic segments inserted into heterochromatin. *Genetics.* 25:618–627.

Earnshaw, W. C., and N. Rothfield. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma (Berl.).* 91:313–321.

Eissenberg, J. C., T. C. James, D. M. Foster-Hartnett, T. Hartnett, and S. C. R. Elgin. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA.* 87:9923–9927.

Eissenberg, J. C., G. D. Morris, G. Reuter, and T. Hartnett. 1992. The heterochromatin-associated protein HP-1 is an essential protein in Drosoph-

ila with dosage-dependent effects on position-effect variegation. *Genetics*. 131:345–352.

Felsenfeld, G., and J. D. McGhee. 1986. Structure of the 30 nm chromatin fiber. *Cell*. 44:375–377.

Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli. 1986. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* 188:613–629.

Gomes, R., R. E. Karess, H. Ohkura, D. M. Glover, and C. E. Sunkel. 1993. Abnormal anaphase resolution (aar): a locus required for progression through mitosis in Drosophila. *J. Cell Sci.* 104:583–593.

Hartmann-Goldstein, I. J. 1967. On the relationship between heterochromatinization and variegation in Drosophila with special reference to temperature sensitive periods. *Genet. Res.* 10:143–159.

Hayden, J. H., S. S. Bowser, and C. L. Rieder. 1990. Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. *J. Cell Biol.* 111:1039–1045.

Huettner, A. F. 1933. Continuity of centrioles in *Drosophila melanogaster*. *Z. Zellforsch. Mikros. Anat.* 19:119–134.

James, T. C., and S. C. R. Elgin. 1986. Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila* melanogaster and its gene. *Mol. Cell Biol.* 6:3862–3872.

James, T. C., J. C. Eissenberg, C. Craig, V. Dietrich, A. Hobson, and S. C. R. Elgin. 1989. Distribution patterns of HP-1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur. J. Cell Biol.* 50:170–180.

John, B. 1988. The biology of heterochromatin. *In* Heterochromatin: Molecular and Structural Aspects. R. S. Verma, editor. Cambridge University Press, Cambridge. 1–147.

Kadonaga, J. T., and R. Tjian. 1986. Affinity purification of sequence-specific DNA-binding proteins. *Proc. Natl. Acad. Sci. USA*. 83:5889–5893.

Kadowaki, T., Y. Shao, and A. M. Tartakoff. 1992. A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. *Proc. Natl. Acad. Sci. USA*. 89:2312–2316.

Karess, R. E., and D. M. Glover. 1989. Rough deal: a gene required for proper mitotic segregation in *Drosophila*. *J. Cell Biol.* 109:2951–2961.

Karr, T. L., and B. M. Alberts. 1986. Organization of the cytoskeleton in early *Drosophila* embryos. *J. Cell Biol.* 102:1494–1509.

Kellum, R., and P. Schedl. 1991. A position-effect assay for boundaries of higher order chromosomal domains. *Cell*. 64:941–950.

Locke, J., M. A. Kotarski, and K. D. Tartof. 1988. Dosage-dependent modifiers of position effect variegation in *Drosophila* and a mass action model that explains their effect. *Genetics*. 120:181–189.

Masumoto, H., K. Sugimoto, and T. Okazaki. 1989. Alphoid satellite DNA is tightly associated with centromere antigens in human chromosomes throughout the cell cycle. *Exp. Cell Res.* 181:181–196.

Masumoto, H., H. Masukata, Y. Muro, N. Nozaki, and T. Okazaki. 1989. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromere satellite. *J. Cell Biol.* 109:1963–1973.

McGhee, J. D., and G. Felsenfield. 1980. Nucleosome structure. *Annu. Rev. Biocem.* 59:1115–1156.

Moroi, Y., C. Peebles, M. J. Fritzler, J. Steigerwald, and E. M. Tan. 1980. Autoantibodies to centromere (kinetochore) in schleroderma sera. *Proc. Natl. Acad. Sci. USA*. 77:1627–1631.

Muro, Y., H. Masumoto, K. Yod, N. Nozaki, M. Ohashi, and T. Okazaki. 1992. Centromere protein B assembles human centromeric α-satellite DNA at the 17-bp sequence, CENP-B box. *J. Cell Biol.* 116:585–596.

Nen, S. S., and A. A. Travers. 1994. HMG-D, the *Drosophila melanogaster* homologue of HMG1 protein is associated with early embryonic chromatin in the absence of histone H1. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 1817–1822.

Palmer, D. K., K. O'Day, and R. L. Margolis. 1990. The centromere specific histone CENP-A is selectively retained in discrete foci in mammalian sperm nuclei. *Chromosoma (Berl.)*. 100:32–36.

Palmer, D. K., K. O'Day, H. Le Trong, H. Charbonneau, and R. L. Margolis. 1991. Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc. Natl. Acad. Sci. USA*. 88: 3734–3738.

Paro, R. 1993. Mechanisms of heritable gene repression during development of *Drosophila*. *Curr. Opin. Cell Biol.* 5:999–1005.

Paro, R., and D. S. Hogness. 1991. The polycomb protein shares a homologous domain with a heterochromatin-associated protein in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 88:263–267.

Paull, T. T., M. J. Haykinson, and R. C. Johnson. 1993. The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. *Genes & Dev.* 7:1521–1534.

Pluta, A. F., N. Saitoh, I. Goldberg, and W. C. Earnshaw. 1992. Identification of a subdomain of CENP-B that is necessary and sufficient for localization to the human centromere. *J. Cell Biol.* 116:1081–1093.

Rattner, J. B., and C. C. Lin. 1985. Radial loops and helical coils coexist in metaphase chromosomes. *Cell*. 42:291–296.

Rattner, J. B. 1991. The structure of the mammalian centromere. *Bioessays*. 13:51–56.

Rattner, J. B., A. Rao, M. J. Fritzler, D. W. Valencia, and T. J. Yen. 1993. CENP-F is a 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil. Cytoskeleton*. 26:214–226.

Saitoh, H., J. Tomkiel, C. A. Cooke, H. Ratrie III, M. Maurer, N. F. Rothfield, and W. C. Earnshaw. 1992. CENP-C, an autoantigen in scleroderma, is a component of the human inner kinetochore plate. *Cell*. 70:115–125.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 11–18.

Santamaria, P. 1986. Injecting eggs. *In* Drosophila: A Practical Approach. D. Roberts, editor. IRL Press, Washington, DC. 159–174.

Sullivan, K. F., and C. A. Glass. 1991. CENP-B is a highly conserved mammalian centromere protein with homology to the helix-loop-helix family of proteins. *Chomosoma (Berl.)*. 100:360–370.

Taylor, J. H. 1991. My favourite cells with large chromosomes. *Bioessays*. 13:479–489.

Widom, J., and A. Klug. 1985. Structure of the 30 A° chromatin filament: x-ray diffraction from oriented samples. *Cell*. 43:207–213.

Williams, B. C., T. L. Karr, J. M. Montgomery, and M. L. Goldberg. 1992. The Drosophila 1(1)zw10 gene product, required for accurate mitotic chromosome segregation is redistributed at anaphase onset. *J. Cell Biol.* 118: 759–773.

Williams, B. C., and M. L. Goldberg. 1994. Determinants of Drosophila ZW10 protein localization and function. *J. Cell Sci.* 107:785–798.

Woodcock, C. L. F., L. Y. Frado, and J. B. Rattner. 1984. The higher order structure of chromatin: evidence for a helical ribbon arrangement. *J. Cell Biol.* 99:42–52.

Yen, T. J., D. A. Compton, D. Wise, R. P. Zinkowski, B. R. Brinkley, W. C. Earnshaw, and D. W. Cleveland. 1991. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *EMBO (Eur. Mol. Biol.) J.* 10:1245–1254.

Yen, T. J., G. Li, B. T. Schaar, I. Szilak, and D. W. Cleveland. 1992. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature (Lond.)*. 359:536–539.

Yoda, K., K. Kitagawa, H. Masumoto, Y. Muro, and T. Okazaki. 1992. A human centromere protein, CENP-B, has a DNA-binding domain containing four potential α helices at the NH_2 terminus, which is separable from dimerizing activity. *J. Cell Biol.* 119:1413–1427.

Zink, D., and R. Paro. 1993. Immunostaining of polytene chromosomes. *Drosophila Information Service*. 72:196.