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Identification of a Nonhistone Chromosomal Protein Associated with Heterochromatin in *Drosophila melanogaster* and Its Gene

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Monoclonal antibodies were prepared against a fraction of nuclear proteins of *Drosophila melanogaster* identified as tightly binding to DNA. Four of these antibodies were directed against a 19-kilodalton nuclear protein; immunofluorescence staining of the polytene chromosomes localized the antigen to the α, β, and intercalary heterochromatic regions. Screening of a λgt11 cDNA expression library with one of the monoclonal antibodies identified a recombinant DNA phage clone that produced a fusion protein immunologically similar to the heterochromatin-associated protein. Polyclonal sera directed against the bacterial *lacZ* fusion protein recognized the same nuclear protein on Western blots. A full-length cDNA clone was isolated from a λgt10 library, and its DNA sequence was obtained. Analysis of the open reading frame revealed an 18,101-dalton protein encoded by this cDNA. Two overlapping genomic DNA clones were isolated from a Charon 4 library of *D. melanogaster* with the cDNA clone, and a restriction map was obtained. In situ hybridization with these probes indicated that the gene maps to a single chromosome location at 29A on the 2L chromosome. This general strategy should be effective for cloning the genes and identifying the genetic loci of chromosomal proteins which cannot be readily assayed by other means.

In eucaryotic organisms, the genetic material exists as a complex between DNA, histones, and nonhistone chromosomal (NHC) proteins. The histones, whose primary structure has been highly conserved over evolution, are the protein components of the nucleosomes and as such are associated with almost all the DNA sequences of the genome. NHC proteins, therefore, are the leading candidates to play specific roles in the organization of higher-order chromatin structure and in the control of gene expression. It appears very likely that specific NHC proteins are involved in the compaction of nucleosomes into domains, the formation of specialized structures such as centromeres and telomeres, and the condensation of the chromatin fiber into the mitotic chromosome structure (9, 12). The presence or absence of various NHC proteins may also be critical in establishing the differences in structure between euchromatin and heterochromatin in the interphase nucleus.

While a great deal of success has been obtained in the characterization of those NHC proteins with a known enzymatic function (16, 24), a systematic biochemical approach to the study of other NHC proteins has proven to be a difficult task. This may be attributed in part to their unusual physico-chemical properties (25), relatively low abundance, and lack of any functional assays. We chose an approach that allowed us to identify NHC proteins of *Drosophila melanogaster* by what is essentially a structural assay. Monoclonal antibodies prepared against fractionated nuclear proteins of *D. melanogaster* embryos are used in immunofluorescence staining of the polytene chromosomes of third-instar larvae of *D. melanogaster*. Those monoclonal antibodies that indicate a protein distribution of interest can be used as probes (66, 67) to identify recombinant cDNA clones from λgt11 cDNA expression libraries. Libraries constructed from RNA from an early stage of embryogenesis may prove to be most effective. These recombinant cDNA probes can be used in the isolation of chromosomal genes from λ Charon 4 genomic DNA libraries (33). The chromosomal gene can be located by in situ hybridization of the genomic DNA clones to polytene chromosomes. In fortunate cases mutants at this locus may be identified from available genetic stocks, while in most cases appropriate deletions for mutant screens can be obtained.

Here we describe the identification of an NHC protein that is primarily located in the heterochromatic regions of the *Drosophila* chromosomes. Monoclonal antibodies directed against this protein were used to identify a cDNA clone from a λgt11 library. We mapped this gene to locus 29A on chromosome 2L. Some functional aspects of this protein based on its distribution along the chromosomes and the amino acid sequence derived from the nucleic acid sequence are also inferred.

MATERIALS AND METHODS

Purification of nuclei from *Drosophila* embryos. Embryos (6 to 18 h old) (1 to 2 kg) were collected from a laboratory population of *D. melanogaster* (Oregon R) maintained at 25°C as described by Elgin and Miller (13). Embryos could be stored for up to 2 years at −80°C without any major alteration of the protein pattern (S. Amero, personal communication). Embryos were dechorionated by stirring in 10 volumes of 2.5% sodium hypochlorite for 3 min followed by extensive washing in tap water and rinsing in distilled water (35). They were collected on Nitex filters and homogenized in cold buffer A (15 mM Tris hydrochloride [pH 7.4], 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*,′-tetraacetic acid], 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin [Sigma Chemical Co., St. Louis, Mo.] per ml, 0.2 Ti units of aprotinin per ml) containing 1.0 M sucrose. The homogenate was filtered through two layers of Miracloth, and the nuclei were isolated as described by Wu et al. (65) except that buffer A was used throughout the procedure. The yield of nuclei per kilogram of embryos was approximately 5×10^{11} nuclei (25 g).

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Fractionation of nuclear proteins. The fractionation of nuclear proteins was carried out as described by Fleischmann and Elgin (manuscript in preparation). Approximately 25 g of nuclei (5×10^{11} nuclei) were homogenized at 4°C with a glass-Teflon homogenizer in 50 ml of buffer B (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 1 mM β-mercaptoethanol, 0.25 M sucrose). The homogenate was centrifuged at 225,000 × *g* for 1 h, and the extraction was repeated with the pellet. The supernatants were pooled, and the pellet was then extracted for 1 h in 50 ml of buffer B containing 0.25 M potassium thiocyanate and centrifuged as described above. This step was repeated once more. The supernatants were pooled, and the pellet was finally extracted twice with 50 ml each of 1.0 M or 2.0 M potassium thiocyanate in buffer B. The pooled supernatants from this step were diluted fourfold in buffer B and concentrated by pressure dialysis with an Amicon YM10 membrane. The retentate was then dissolved in 50 ml of buffer C (4 M urea, 1 M NaCl, 10 mM potassium phosphate, pH 6.8). An Ultrogel HA (LKB Instruments, Rockville, Md.) column (5 by 4 cm) was prepared in buffer C, and the retentate was loaded onto the column. The flowthrough fraction was collected and reapplied to the column. The flow rate was 30 ml/h. The column was washed extensively with buffer C and was developed with a step gradient of 100 ml each of 0.1, 0.4, and 1.0 M potassium phosphate (pH 6.8) in buffer C. The eluted proteins were dialyzed against 10 mM acetic acid and stored as lyophilized powder.

Preparation of antibodies. Proteins separated on acetic acid-urea (39)-polyacrylamide gels (acrylamide-bisacrylamide, 30:0.2, wt/wt) were stained briefly with Coomassie blue and washed for 2 h in 10% methanol–7.5% acetic acid (destain). The gel was then treated with 2% glutaraldehyde solution for 1 h and washed in destain for two more hours with three to four changes. Typically, Coomassie blue-staining bands (approximately 1 to 2 μg of protein each) from two 4-mm-wide lanes of a gel (15 by 15 by 0.1 cm) were excised after glutaraldehyde fixation and were ground wet into a paste. This was then freeze-dried, suspended in Fruend adjuvant, and used as an immunogen (22). The booster injections were given after 21 days. Immune sera from the mice were collected 10 days after the booster injection. Preimmune serum and immune serum from the same mouse were compared by the immunofluorescence staining assay on polytene chromosomes (53) to identify antigens that had an active association with chromosomes.

After the identification of mice that gave a response of interest, a further booster injection was given, and the animals were sacrificed 72 to 84 h later. Spleen cells were prepared from each mouse, and they were fused to Sp2/0 Ag 14 myeloma cells at a ratio of 1:10 with 50% polyethylene glycol 1450 as described by Oi and Herzenberg (38). Cells after fusion were seeded into 10 microtiter plates that had a feeder layer of 10^3 peritoneal cells per well. Media and supplements used in these experiments were as described by Siraganian et al. (56). Our fusions gave 40 to 60% growth positives. Primary screening of the hybridomas was performed by an enzyme-linked immunosassay. Fractions from the Ultrogel HA column were used as antigens at a concentration of 1 μg/ml. The proteins were fixed onto the microtiter plates with carbodiimide (59). A biotin-streptavidin-horseradish peroxidase system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used to identify the positive clones as described by the manufacturer. Positive clones from this screen were then transferred to a 24-well plate and grown in the hybridoma complete medium containing 75 μg of endothelial cell supernatant growth factor (Collaborative Research, Inc., Waltham, Mass.) per ml. After cells attained confluency, cell supernatants from these wells were screened on a Western blot (60) with proteins from the Ultrogel HA fraction. Supernatants (50 to 100 μl) from clones that produced antibodies against the protein antigen were further screened by the immunofluorescence assay (53). A fluorescein-conjugated antimouse second antibody (Miles Laboratories, Inc., Elkhart, Ind.) that recognizes mouse immunoglobulin G, immunoglobulin M, and immunoglobulin A molecules was used in this screening. Hybridoma supernatants that showed a positive signal on all three assays were cloned by limiting dilution or soft-agar techniques or both (14). After identification of the monoclonal cell lines of interest, the cells were used in the production of ascites. Clarified ascites fluids were stored at −80°C (40).

Immunofluorescence analysis. The distribution patterns obtained with the preimmune serum, immune serum, and monoclonal antibodies were determined by the methods of Silver and Elgin (53) with the modification that 0.05% Tween 20 was present in all solutions throughout the procedure and 10% bovine calf serum was present in the antibody solutions.

Screening of λgt11 cDNA expression library. The λgt11 cDNA library used was originally constructed in λgt10 and subsequently recloned into λgt11 by using *Eco*RI sites (M. Philip and D. Brutlag, personal communication). The procedure for screening the λgt11 library was similar to that described by Huynh et al. (23). We used *Escherichia coli* Y1088 on LB plates containing 100 μg of ampicillin per ml. Isopropyl-β-D-thiogalactopyranoside induction was for 10 to 12 h. After induction, the nitrocellulose paper was incubated for 2 h at 37°C in Tris-buffered saline (50 mM Trishydrochloride [pH 7.5], 150 mM NaCl) (TBS) containing 10% bovine calf serum, 1% hemoglobin, 100 μg of bovine gamma globulins per ml, 0.2% sodium azide, and 0.5% Tween 20 (TBS complete). Monoclonal antibody (C1A9), often as clarified ascites fluid, was diluted 1:20 in the TBS complete medium, and the filters were incubated at 37°C for 2 h. They were then washed three times for 10 min each in excess TBS with 0.5% Tween 20, or until all the hemoglobin had come off the filters. The second antibody was ^{125}I-labeled F(ab')$_2$ fragment against mouse immunoglobulin at a dilution of 1:500 (approximately 10^5 cpm/ml) in TBS complete medium. The filters were incubated for 1 h at 37°C and washed as before. Filters were blotted dry and exposed to Kodak X-Omat AR film with Du Pont Cronex Lightning-Plus intensifying screens at −80°C for 12 to 24 h. Positive plaques were rescreened as described above.

Production of polyclonal antisera against fusion protein. A positive plaque that produced a fusion protein antigenically similar to C1A9 antigen was lysogenized, and the bacterial proteins were isolated after induction of a 500-ml liquid culture (23). Fractionation of the bacterial lysate to enrich for the fusion protein was done as described by Muller-Hill and Kania (37) except that an Ultrogel HA column was used instead of a phosphocellulose column. Proteins that eluted at 0.2 M potassium phosphate (pH 6.8) were precipitated with 20% trichloroacetic acid, and a small sample was separated on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The position of the fusion protein on the gel was identified by Western blot analysis of a portion of the gel with C1A9 antibody. The Coomassie blue-stained band was excised from the gel and used to immunize mice as described above. After booster injections, the polyclonal serum was collected from the animal.

Gel electrophoresis. (i) Protein gels. Gradient (15% or 6 to 18%) 15% SDS-polyacrylamide (30:0.8) gel electrophoresis was performed as described previously (27). For precise molecular weight determinations, nuclear proteins were also analyzed along with molecular weight standards on 10 and 12% urea-SDS-polyacrylamide (30:0.8) gels with a neutral pH phosphate buffer (pH 7.3) system (7; H. Ley, Focus **6**:5, 1984). The 6 M urea–5% acetic acid–polyacrylamide (30:0.8 or 30:0.2) gels used were similar to the gels of Panyim and Chalkley (39) with minor modifications (2). Some urea-acetic acid gels contained up to 0.4% Triton DF16. The Coomassie blue-stained gel strips from urea-acetic acid gels were analyzed on second-dimension SDS-polyacrylamide gels as described by Allis et al. (2).

(ii) RNA gels. Total RNA was separated on 1% agarose gels containing 2.2 M formaldehyde and 10 μg of ethidium bromide per ml as described by Lehrach et al. (30) except that MOPS (3-[morpholino]propanesulfonic acid)-acetate buffer (pH 6.8) was used.

(iii) DNA gels. Restriction endonuclease digests of phage and *Drosophila* genomic DNA were separated on 1% agarose gels in a Tris acetate-EDTA buffer system (51). DNA-sequencing gels were made as described by Sanger and Coulson (48).

Southern, Northern, and Western blot analysis. Southern transfers were done by the method of Southern (58) except that the DNA was depurinated (61) and 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was used as the transfer buffer. For Northern transfers, formaldehyde-containing RNA gels were blotted in 20× SSC onto BA 85 (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) nitrocellulose immediately after electrophoresis. After fixation of nucleic acids to the paper, they were washed in distilled water and hybridized to nick-translated DNA (44) probes as described by Church and Gilbert (11). Western blot analyses of SDS-protein gels were performed by the method of Towbin et al. (60) except that 0.1-μm-pore-size nitrocellulose filters (Schleicher & Schuell) were used in Tris-glycine transfer buffer containing 10% methanol. After transfer, nitrocellulose sheets were processed for antibody screening as described above for λgt11 screening. We also used a biotin streptavidin-alkaline phosphatase system (Bethesda Research Laboratories) to immunostain Western blots according to the directions of the manufacturer.

Isolation of nucleic acids. Rapid lysate and large-scale preparations of phage DNA were done as described previously (23). Plasmid DNA was isolated by the method of Birnboim (6); high-molecular-weight *Drosophila* genomic DNA was isolated from 6- to 18-h-old embryos by the method of Wu et al. (65). Total RNA from 6- to 18-h-old embryos was isolated by the lithium chloride-urea extraction procedure (3). The final RNA pellet was dissolved in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA (TE buffer) containing 0.2% lauryl sarcosyl. Cesium chloride was added to 5.7 M, and the RNA was pelleted at 50,000 rpm for 12 h in a type 865.1 rotor (Du Pont Sorvall Inc., Wilmington, Del.). The RNA pellet was dissolved in TE buffer and precipitated with 2 volumes of ethanol. The RNA was reprecipitated until no more cesium chloride remained in the pellet.

DNA sequencing. The chemical sequencing of the λgt10 and λgt11 cDNA inserts was performed by the method of Maxam and Gilbert (34). Dideoxy sequence analysis of the cDNA insert was performed by using synthetic primers to the *Eco*RI site of the λgt11 vector (New England BioLabs, Inc., Beverly, Mass.), the *Eco*RI and *Hin*dIII sites of the pBR322 vector (Pharmacia Inc., Piscataway, N.J.), and a 20-mer 5′-$_{p}$CCGAGCTCTCAGGGTTGTCG$_{OH}$ complementary to nucleotides 363 to 383 as described by Wallace et al. (62) and Hattori and Sakaki (20). Either both strands were sequenced or one strand was sequenced by both methods over the entire cDNA.

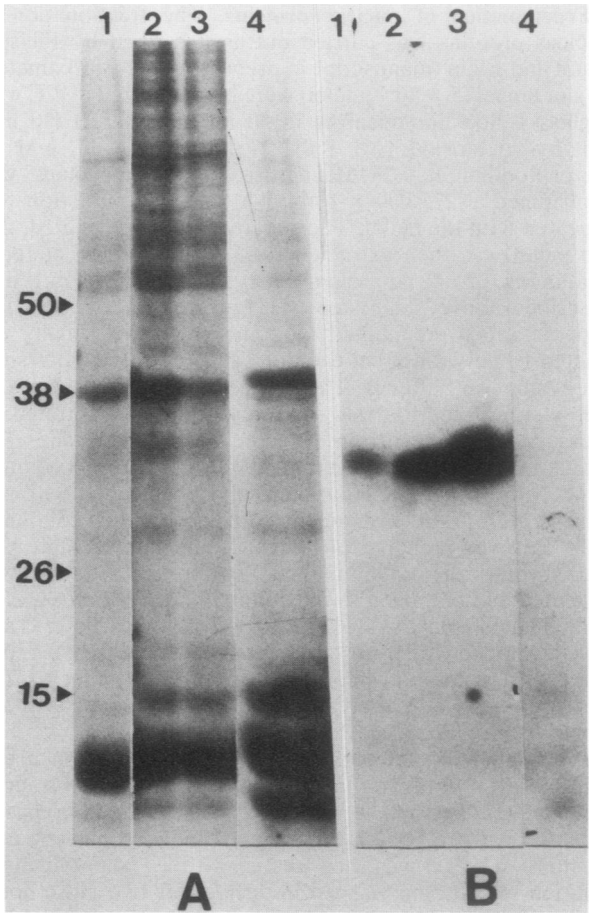


FIG. 1. Extraction of C1A9 antigen from nuclei with potassium thiocyanate. The nuclear proteins extracted with potassium thiocyanate were analyzed in duplicate on a 15% SDS-polyacrylamide gel (25 to 50 μg of protein per lane) with discontinuous buffer systems (27) as described in Materials and Methods. After electrophoresis, proteins from one half of the gel were blotted onto a nitrocellulose filter and probed with monoclonal antibody C1A9 and ^{125}I-labeled anti-mouse immunoglobulin antibodies for C1A9 antigen detection. (A) Coomassie blue-stained half of the gel; (B) autoradiograph of the Western blot. Lanes: 1, 0.25 M potassium thiocyanate fraction; 2, 1.0 M potassium thiocyanate extract; 3, 2.0 M potassium thiocyanate extract; 4, 0.4 N H_2SO_4-soluble nuclear proteins. The molecular weight ($\times 10^3$) standards are shown on the left.

Screening of Charon 4 genomic and λgt10 cDNA libraries. A Charon 4 *Drosophila* (Canton S) genomic library (33) and a λgt10 cDNA library (41) constructed from poly(A)-containing RNA isolated from 0- to 3-h-old *D. melanogaster* (Oregon R) embryos were screened by using a subclone in plasmid pBR322 of the λgt11 cDNA as a probe. Plaque lifting and processing were as described previously (5), while hybridizations were performed as described above.

In situ localization of the gene encoding the C1A9 antigen. In situ localization of the gene for the C1A9 antigen on the polytene chromosomes was done by a procedure communicated to us by William Engels (University of Wisconsin),

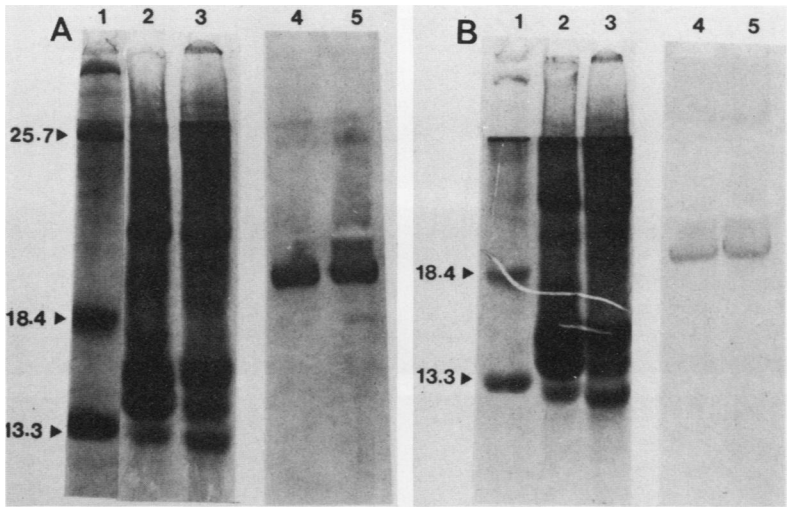


FIG. 2. Urea-SDS-polyacrylamide (10% in panel A and 12% in panel B) gel electrophoretic analysis of C1A9 antigen with a continuous neutral buffer system (7; H. Ley, Focus 6:5, 1984). Photographs of both Coomassie blue-stained gels (lanes 1, 2, and 3) and immunostained filters (lanes 4 and 5 corresponding to lanes 2 and 3 of the Coomassie blue-stained gel) are shown. Lane 1 shows molecular weight standards: γ-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; and lysozyme, 13,300. Other higher-molecular-weight standards did not migrate into the resolving gel and can be seen in the stacking gel. Lanes 2 and 3 were loaded with the same protein samples as in lanes 2 and 3 of Fig. 1. After electrophoresis, proteins from one half of each gel were transferred to nitrocellulose filters (60). These filters were then processed for immunostaining with C1A9 monoclonal antibodies. Biotinylated anti-mouse immunoglobulin antibodies and alkaline phosphatase-conjugated streptavidin (Bethesda Research Laboratories) were used for detection of C1A9 antigen.

using a biotinylated (44) cloned genomic DNA probe for the C1A9 antigen. A polyalkaline phosphatase enzyme-linked immunoassay (Bethesda Research Laboratories) was used to identify the hybrids.

RESULTS

Identification of heterochromatin-associated NHC protein. Nuclei were isolated from 6- to 18-h-old embryos in the presence of protease inhibitors. Proteins were released from the nuclei with increasing concentrations of potassium thiocyanate (KSCN) (Fleischmann and Elgin, in preparation). In this paper we describe the identification of a chromosomal protein that was present in 1.0 and 2.0 M potassium thiocyanate fractions. Electrophoretic analysis of the proteins from the various fractions on SDS-Tris-glycine-polyacrylamide gels (Fig. 1A) illustrates the fractionation pattern obtained. Antibodies were produced in mice against a selection of proteins released from the nuclei at 2.0 M KSCN.

A group of four hybridoma cell lines from one fusion, designated here as C1A9, C9A10, C4D12, and C9C7, produced monoclonal antibodies that recognized a particular nuclear protein. The immunogen was a protein that was released from the nuclei at 1.0 or 2.0 M potassium thiocyanate and eluted from the hydroxylapatite column at 0.1 to 0.4 M phosphate concentration. Western blot analysis of nuclear proteins with these monoclonal antibodies confirmed that this protein (designated as C1A9 antigen) was indeed released from chromatin primarily at higher concentrations of KSCN (Fig. 1B). This protein migrated more slowly than histone H1 on urea-acetic acid gels (data not shown); it migrated with an apparent molecular weight of 34,000 on SDS-containing gels. Unlike histones, this protein was not released by extraction of chromatin with 0.4 N H_2SO_4 (Fig. 1, lane 4).

It is known that some chromosomal proteins of *D. melanogaster* migrate aberrantly on SDS-polyacrylamide gels when discontinuous buffer systems are used (27). Histone H1 migrates at an apparent molecular weight of 38,000, while the actual molecular weight calculated from amino acid sequence data is 21,990 (63). To obtain a more precise molecular weight for C1A9 antigen, we used a urea-SDS-polyacrylamide gel which employs a neutral continuous buffer system. In this gel system histone H1 from *D. melanogaster* migrated with a molecular weight of approximately 22,000. After electrophoresis, the proteins from the gels were transferred to nitrocellulose filters, and the filters were probed with monoclonal antibodies directed against C1A9 antigen. The exact position of C1A9 antigen was identified from the positive signal on the nitrocellulose paper. The molecular weight of the C1A9 antigen was calculated from known molecular weight standards electrophoresed on the same gel (Fig. 2A and B). Using this gel system, it was found that the C1A9 antigen migrates at a molecular weight of approximately 19,000.

Immunofluorescence analysis on polytene chromosomes from third-instar larval salivary glands with the monoclonal antibodies that recognized C1A9 antigen revealed that the protein is located in well-defined regions of the chromosomes, previously identified by cytological techniques as constitutive heterochromatin (Fig. 3). These include the chromocenter and the intercalary heterochromatin at cytological map position 35 to 36 on chromosome 2L (18, 46). Fluorescence elsewhere along the euchromatic arms was minimal. Slight differences in the staining pattern of intercalary heterochromatin were observed between monoclonal antibodies C1A9 and C9A10 on the one hand, and C4D12 and C9C7 on the other. Representative staining patterns are shown in Fig. 3 A, B, and C.

Salivary gland polytene chromosome squashes were prepared after fixation in acetic acid or in formaldehyde solutions (54); acetic acid is known to solubilize histone H1 and some other chromosomal proteins. Fixation by either procedure resulted in the same staining pattern of the

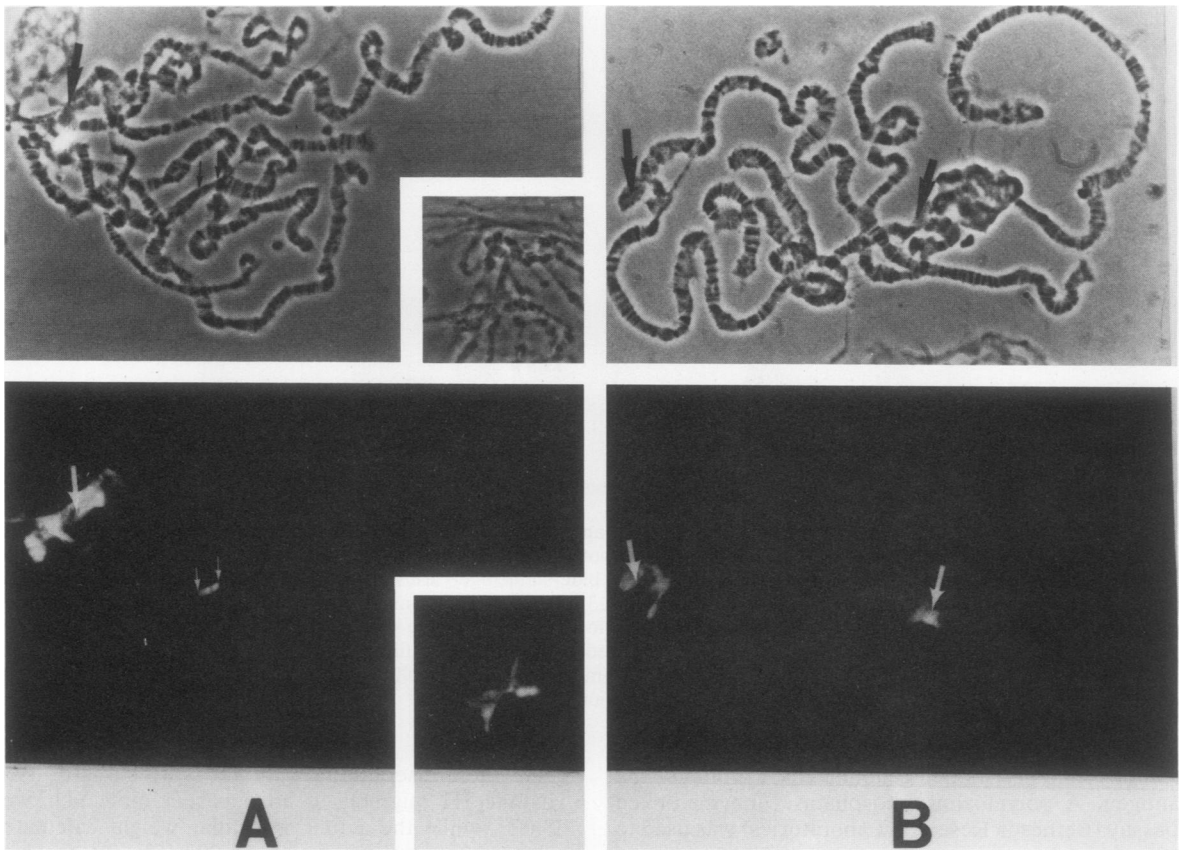
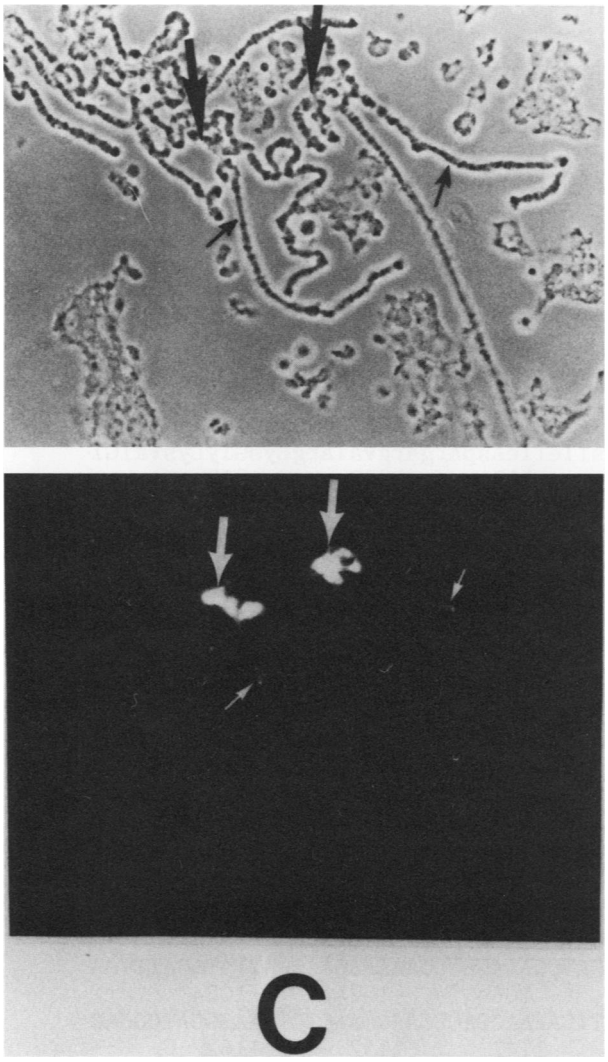


FIG. 3. Immunofluorescence staining of polytene chromosomes. Three different monoclonal antibodies, C1A9 (A), C9C7 (B), and C9A10 (C), directed against the same nuclear protein were used in the immunofluorescence assay on third-instar larval polytene chromosomes. Note that the staining is primarily limited to the chromocenter (thick arrows) and a few bands along the arms. In panel B, the chromocenter is split into two halves, and staining is limited to these regions. In panels A and C, prominently stained regions on the chromosome arms are clearly visible, particularly at locus 35 to 36 on 2L (thin arrows). In all cases the upper panels show the phase-contrast pictures, while the lower panels show the epifluorescence of fluorescein isothiocyanate-conjugated second antibody under UV light. The inset in panel A shows a preferential immunofluorescence staining of β-heterochromatin by C1A9 monoclonal antibody.

chromocentric heterochromatin of the polytene chromosomes. Based on the KSCN extraction of *Drosophila* embryo nuclei and the retention of this antigen after acetic acid fixation of the polytene chromosomes, it appears that C1A9 protein is tightly bound to the chromatin. In all staining experiments with C1A9 or other antibodies against the same protein, we did not observe any cytoplasmic staining, indicating that the C1A9 antigen in the cell is exclusively located on the chromosomes.

Isolation of cDNA clone that codes for C1A9 antigen. The cDNA library used in this study was prepared from poly(A)$^+$ RNA isolated from 1.5 to 5-h-old *D. melanogaster* embryos (M. Philip and D. Brutlag, personal communication). The fact that heterochromatinization occurs ca. 2 h after fertilization suggested a higher rate of synthesis of C1A9 antigen at this stage of embryogenesis. Screening of 2×10^5 recombinant DNA phage from the λgt11 cDNA library with the C1A9 monoclonal antibody identified two plaques that on isopropylthiogalactopyranoside induction produced *lacZ* fusion proteins that were antigenically similar to the heterochromatin-associated C1A9 antigen. The molecular weight of one of the fusion proteins was calculated from SDS gels to be 135,000. This indicates an approximately 600- to 700-nucleotide-long open reading frame in the cDNA insert. To positively identify this cDNA clone, we raised a polyclonal antiserum against the fusion protein. The antiserum was used in a Western blot analysis in which the *lacZ* fusion protein and *Drosophila* nuclear proteins were separated in parallel on a 6 to 18% SDS-polyacrylamide gradient gel. Monoclonal antibodies C1A9 and C9A10 reacted to both the galactosidase fusion protein (135 kilodaltons) and the original *Drosophila* nuclear antigen (Fig. 4A and B). Polyclonal antiserum against the fusion protein recognized the fusion protein and a second bacterial protein (Fig. 4C, lane 1). It also reacted very specifically to the *Drosophila* heterochromatin-associated nuclear protein, indicating that the fusion protein and the nuclear protein share very specific common antigenic determinants. Of the four monoclonal antibodies which recognized the nuclear protein, both C1A9 (Fig. 4B) and C9A10 (Fig. 4A) recognized the fusion protein, while the C9C7 and C4D12 antibodies did not. This suggests that C9C7 and C4D12 recognize a different antigenic determinant that C1A9 and C9A10.

Nucleotide sequence of cDNA clone. Total RNA isolated from 6- to 18-h-old embryos was separated on an agarose gel under denaturing conditions and probed with the nick-translated cDNA clone. A single species of mRNA of 1.0 to 1.1 kilobases hybridized to the cDNA (data not shown). Comparison of the sizes of the RNA on the Northern blot and the cDNA insert revealed that the cDNA insert obtained



from the λgt11 lacks approximately 300 nucleotides at its 5′ end. To obtain a full-length cDNA clone, we screened a λgt10 cDNA library constructed from poly(A)-containing RNA isolated from 0- to 3-h-old *Drosophila* embryos. The λgt10 cDNA library was constructed (41) by using GC tailing at the 5′ end on the first synthesized DNA strand to maximize the yield of 5′ full-length cDNA clones (29). A number of positive phage plaques were identified, and the lengths of the cDNA inserts contained within them were calculated. The largest λgt10 cDNA clone that carried a 1.2-kilobase insert was chosen to study in detail.

To understand the nature of the heterochromatin-associated protein, we sequenced the λgt11 and λgt10 cDNA clones by both chemical (34) and dideoxy (20, 49, 62) sequencing techniques. The cDNA inserts from both λgt10 and λgt11 were subcloned into pBR322 at the *Eco*RI site. The orientations of both clones were identified by the polyadenylation consensus sequence (at bases 1146 to 1152 in λgt10) and the poly(A) tail (data not shown) at the 3′ end. The protein-coding strand was identified by probing a Northern blot with single-stranded M13 probes (data not shown), and the correct reading frame was determined in λgt11 cDNA by the Glu-Phe amino acids coded in frame by the regenerated *Eco*RI site used in the original ligation reaction.

The nucleic acid sequence of the λgt10 cDNA clone contains a 349-base-pair (bp)-long untranslated 5′ sequence before a 483-bp-long open reading frame that codes for a protein with a molecular weight of 18,101 (Fig. 5). At the 3′ end, there is a 338-bp-long untranslated sequence before the poly(A) tail. The untranslated regions at both ends contain multiple termination codons on all three reading frames. The translational initiation ATG codon and the surrounding sequence 5′-AAAATGG-3′ are in general agreement with consensus sequence 5′-ACCATGG-3′ for the "scanning model" of initiation of translation of eucaryotic messengers proposed by Kozak (26). All of the 483-bp protein-coding sequence and the 338-bp 3′-untranslated sequences are present in the λgt11 cDNA clone. However, −5 upstream from the ATG initiation codon, 13 nucleotides are different in the λgt10 and λgt11 cDNA inserts (see λgt11 cDNA sequence in Fig. 5). This is possibly artifactual since the λgt11 cDNA library was constructed from a λgt10 library by digestion of the whole library with *Eco*RI and subsequent mass isolation of the inserts followed by ligation into the *Eco*RI site of λgt11. Except for this difference and the regenerated *Eco*RI recognition sequence in the λgt11 insert, the nucleic acid sequences from both λgt10 and λgt11 cDNA inserts are identical.

The amino acid sequence derived from the DNA sequence (Fig. 5) revealed some interesting features of the protein.

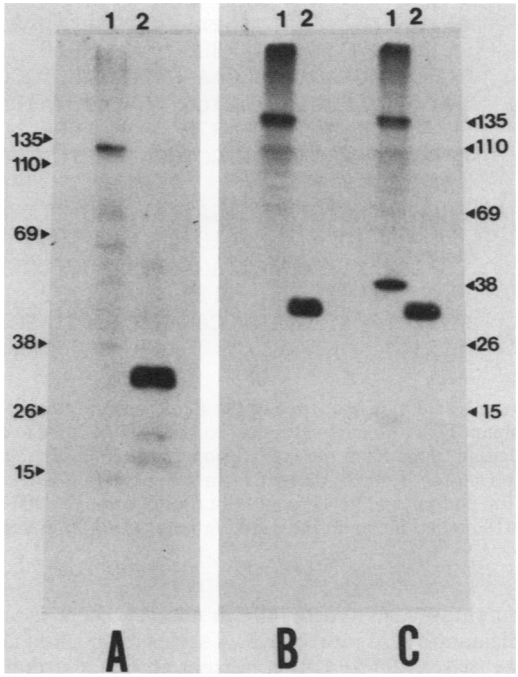


FIG. 4. Immunological identification of the recombinant DNA-derived *lacZ* fusion protein. A 6 to 18% SDS-polyacrylamide gel (discontinuous buffer system) was used to separate partially purified β-galactosidase fusion protein from a lysogenized recombinant DNA phage (lane 1) and *D. melanogaster* nuclear proteins (lane 2, 2.0 M potassium thiocyanate extract). The proteins were then blotted onto nitrocellulose and probed with the monoclonal antibody C9A10 (A) or C1A9 (B) directed against the nuclear protein or with an anti-*lacZ* fusion protein antiserum (C). The nuclear protein sample loaded on this gel was the same as that in Fig. 1, lane 3. The gel shown in panel A was run separately from that in shown panels B and C. Molecular weight standards ($\times 10^3$) are indicated in each case.

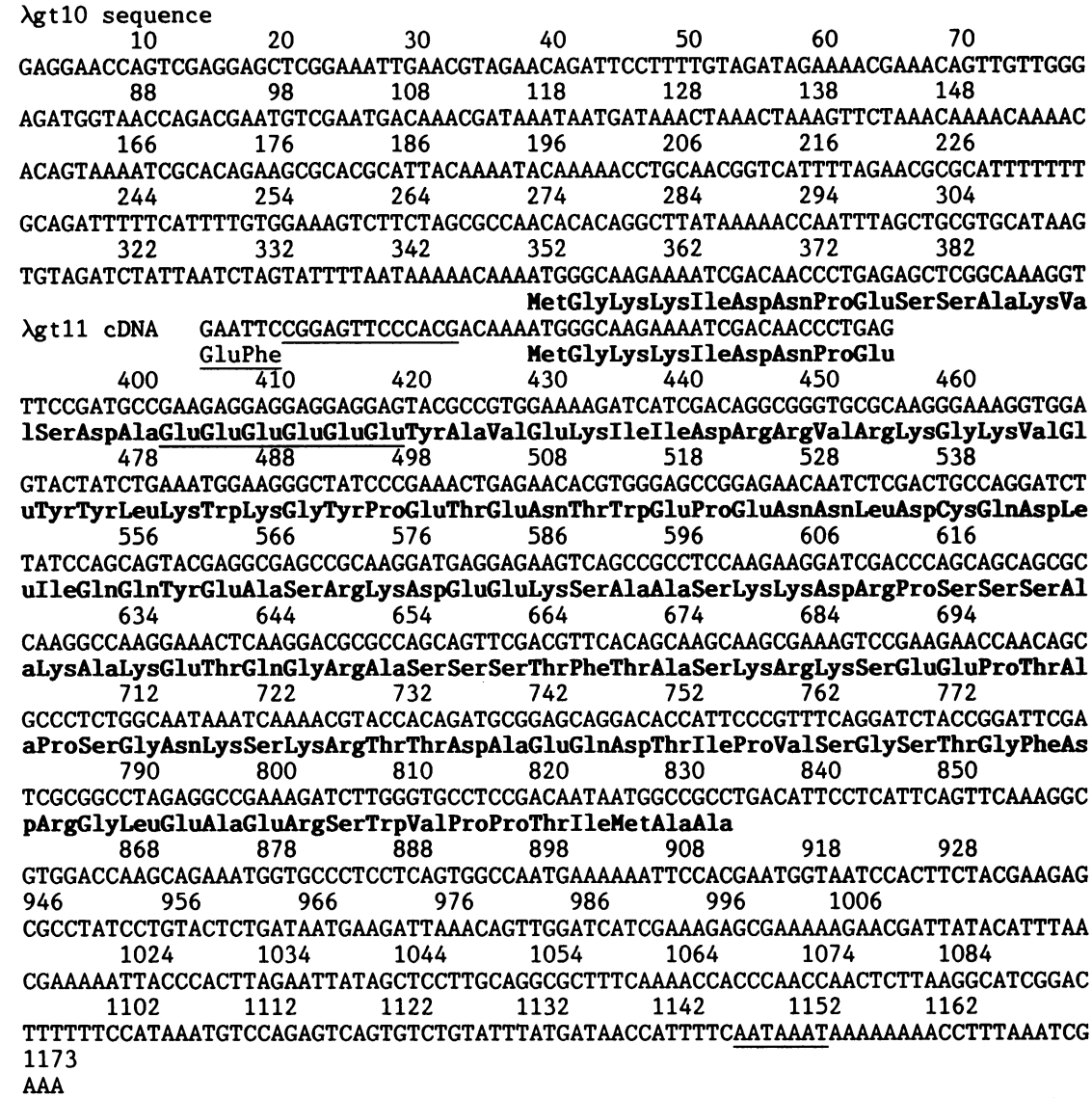


FIG. 5. Nucleotide sequence of the *Eco*RI insert in λgt10 recombinant DNA phage. The nucleotide sequence of the *Eco*RI insert in λgt11 recombinant DNA phage is identical to the λgt10 cDNA insert except for base pairs 1 to 345. Hence, only the first 50 nucleotides with the open reading frame from the *lacZ* fusion and the Glu-Phe amino acids coded by the *Eco*RI linkers of the λgt11 insert are shown (see text). An amino acid sequence is derived from the nucleotide sequence. Only one of the reading frames is shown. The other two reading frames have many stop codons and hence could not produce the 135,000-dalton *lacZ* fusion protein seen in λgt11 phage. The polyglutamic acid stretch at 400 to 418 nucleotides and the putative poly(A) addition consensus sequences are underlined.

The protein is very hydrophilic in nature. There is a basic-to-acidic amino acid ratio of 1.2. A series of six glutamic acid residues (bases 400 to 418) is present among a stretch of 20 amino acids (bases 392 to 463) that has the potential to fold in to an α-helical structure. The molecular weight of the protein derived from the DNA sequence is 18,101, which is in agreement with the molecular weight of the C1A9 antigen obtained from urea-SDS-polyacrylamide gels (7; H. Ley, Focus **6**:5, 1984).

Isolation of a genomic DNA clone and in situ localization of the gene coding for the heterochromatin-associated protein. We screened a Charon 4 genomic library for DNA fragments that contain the C1A9 antigen gene. A number of overlapping DNA fragments that hybridize to the λgt11 cDNA clone were identified. Two of these clones were characterized in detail. A partial restriction map of the two genomic clones (Charon 4 HCDm 7 and 1.4) and the localization of the cloned cDNA sequences are shown in Fig. 6. Northern blot analysis of embryo RNA with Charon 4 HCDm 7 as a probe indicated the presence of a second gene with a less-abundant transcript of approximately 1.3 kilobases (data not shown). At present we do not know the functional role of the protein encoded by this transcript. The genomic DNA clones Charon 4 HCDm 1.4 and 7 were used to probe salivary gland polytene chromosomes in situ. A single site that mapped to 29A on the second chromosome was identified as the chromosomal location of the gene (Fig. 7). The hybridization signal indicated a broad region at 29A, raising the possibility that there may be one or two copies of this gene or a similar gene at this location.

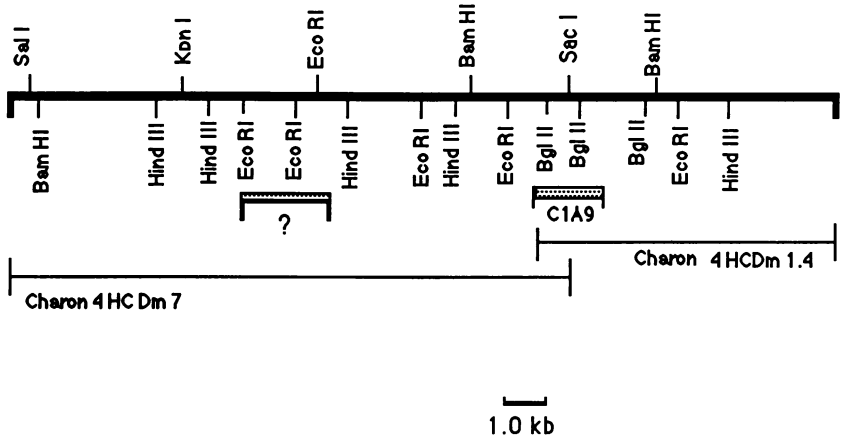


FIG. 6. Partial restriction map of the two overlapping genomic DNA clones isolated from a Charon 4 genomic library. The solid bars represent the two transcription units. ? shows the position of the uncharacterized transcription unit which lies toward the 3' end of C1A9 transcription unit. kb, Kilobases.

DISCUSSION

Our understanding of the structural and functional roles of chromosomal proteins has been greatly aided by the development of immunological techniques to localize protein antigens on eucaryotic chromosomes (1, 17, 52, 53) with very specific antisera. A necessary requisite to such an analysis is the availability of monospecific antibodies to various chromosomal proteins. Recently, it has been possible to produce monoclonal antibodies from impure antigens, as has been done for NHC proteins of the *D. melanogaster* cell line Kc (50). One of the drawbacks of this approach is the fact that the use of crude protein preparations as antigens often results in the production of a large number of monoclonal antibodies that recognize a small number of major antigenic components in the preparation. To overcome this drawback, the antigen used in our study was prepared from gel electrophoretically separated proteins. Our experimental approach is designed for proteins that are present in low amounts and that are difficult to purify to homogeneity by conventional biochemical techniques. Consequently, we used 1 to 2 μg of protein per injection for immunization. Hydroxylapatite fractionation of nuclear proteins was chosen primarily because separation can be achieved in the presence of denaturing solvents, which are necessary to maintain these proteins in solution.

We identified four different monoclonal antibody-secreting cell lines from a single fusion. In an immunofluorescence assay, all four monoclonal antibodies recognized an antigen that is primarily located at the chromocenter. A small, but consistent number of chromosomal sites, notably region 35 to 36 on the 2L chromosome, were also stained. A detailed analysis of all the regions that are stained by this assay indicates that the antigen is concentrated at previously identified centromeric and intercalary heterochromatic sites (T. C. James and V. Dietrich, unpublished data).

Heterochromatin in eucaryotic cells can be classified into two types, constitutive and facultative. Constitutive or obligatory heterochromatin is commonly found in the form of comparatively short stretches of highly condensed material that always occur at the same location in both members of a homologous pair of chromosomes. It is found at centromeres, usually at both sides of the kinetochore. At interphase the regions of constitutive heterochromatin may remain aggregated to form chromocenters. Obligatory heterochromatin in *D. melanogaster* occupies approximately 25 to 50% of all major chromosome arms and the Y chromosome. It is also interspersed in many euchromatic sites. Cytological distinctions sometimes have been drawn between the material of the chromocenter (α-heterochromatin) per se and the condensed regions immediately adjacent which connect the chromocenter to the chromosome arms (β-heterochromatin). While α-heterochromatin is underreplicated, β-heterochromatin, which has a banded structure, appears to replicate with euchromatin and also appears to be transcribed at low levels (28). In the immunofluorescence assay with C1A9 antibody we observed a more intense staining in the β-heterochromatin than in the α-heterochromatin (Fig. 3). This may reflect the DNA and protein content of the respective regions.

The C1A9 antigen, like *Drosophila* histone H1, migrates aberrantly on SDS-polyacrylamide gels that use discontinuous buffer systems (27). The apparent molecular weight of 34,000 calculated for C1A9 based on its migration on these gels is approximately twice the absolute molecular weight obtained from the amino acid sequence data. However, in a urea-containing SDS gel with a continuous neutral buffer system, both C1A9 antigen and histone H1 migrated very close to their absolute molecular weights derived from nucleic acid or amino acid sequence data at 19,000 and 22,000, respectively. The exact reasons for the anomalous migration of some proteins on SDS gels is not clearly understood. The anomalous migration may be due to specific secondary structures or to an uneven distribution of charge throughout the protein. The presence of urea and SDS in the gel at neutral pH presumably results in complete denaturation of these proteins.

There appear to be multiple interdependent factors that determine heterochromatinization, including the nucleotide sequences in the heterochromatic DNA and the various chromosomal proteins that bind to them. Analysis in *D. melanogaster* has identified several AT-rich satellite DNAs present in the constitutive heterochromatin. The satellite DNA with a bouyant density of 1.688 (8) has been shown to bind to at least two proteins in a DNA filter binding assay (21). In another study, it was shown that an NHC protein called D1 (1, 31) specifically binds to highly AT-rich DNA. Using immunological techniques, Will and Bautz (64) have identified a protein of approximately 38,000 daltons (based on migration of the protein in SDS gels with the discontinu-

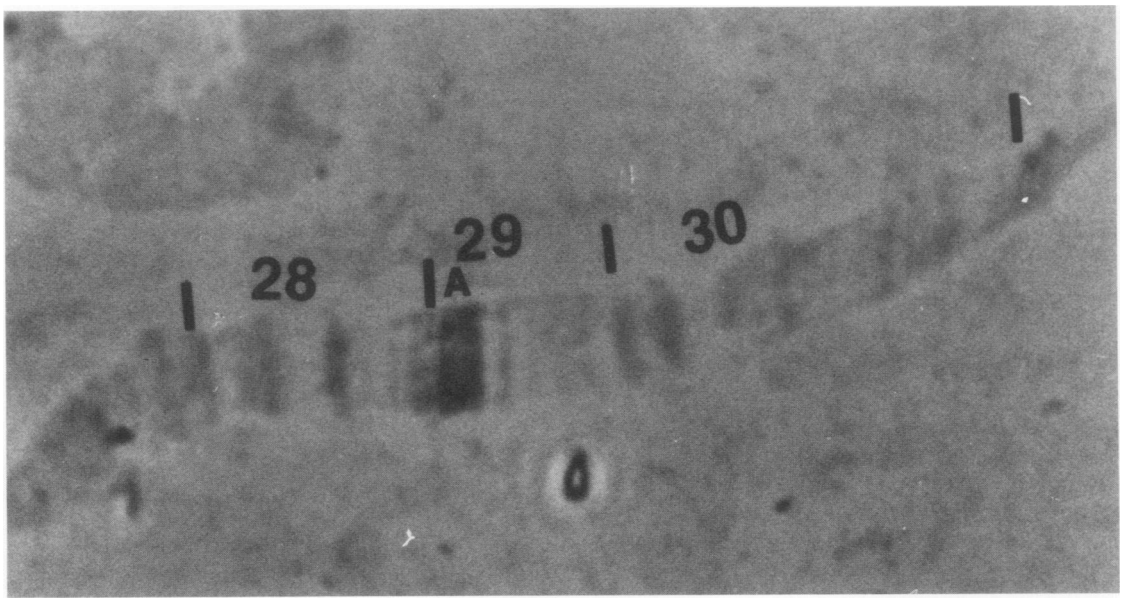


FIG. 7. In situ localization of the gene coding for the C1A9 antigen. A genomic DNA clone (Charon 4 HCDm 7) isolated from a Charon 4 library was nick translated (44) with biotinylated dUTP and used to probe a polytene chromosome squash as described in Materials and Methods. The hybridization signal is located exclusively at cytological map position 29A on the 2L chromosome.

ous buffer system) as a constituent of heterochromatin. These authors prepared antisera against two chromosomal protein fractions obtained from a hydroxylapatite column. Both antisera showed bright fluorescence staining at the chromocenter. A number of other bands along the chromosome arms were also stained. Because of the impurities in the antigen the authors could not conclusively attribute the various staining patterns obtained in this experiment to any specific antigen, although the 38,000-dalton protein was one of the major components immunoprecipitated from that fraction. It is possible that the antigen we identified in the heterochromatic regions is identical to that described by Will and Bautz (64). The facts that the antigen described here and that described previously both elute from hydroxylapatite columns at 100 to 150 mM phosphate concentrations and that both migrate similarly on urea-acetic acid and SDS-polyacrylamide gels with discontinuous buffer systems strongly suggest that they are the same antigen.

The C1A9 antigen appears to be very hydrophilic and has a high predicted helical content similar to high-mobility group proteins (4, 10). In addition, polyglutamic acid stretches (15) and long 3′-untranslated regions (G. H. Dixon, B. T. Pentecost, K.-L. De. Lee, and J. M. Wright, Abstr. Int. Symp. Chromatin Struct. Function, Camarino, Italy, 1985, p. 61–62) similar to those seen in the C1A9 antigen mRNA have also been observed in high-mobility group proteins. Recently mRNA for another DNA-binding protein, GCN4 in yeasts, has been characterized (36). GCN4 protein synthesis is regulated at the translational level, and its mRNA has a 600-nucleotide-long untranslated 5′ noncoding region. If C1A9 synthesis is also regulated at the translational level, the long untranslated 5′ sequences of C1A9 antigen mRNA may have a functional role. Comparison of the nucleic acid sequence to Genebank sequences and the protein sequence to the protein sequence data bank did not show any significant similarities to other known sequences.

In both drosophila (32) and mice (47) the presence of a euchromatic gene in close proximity to the constitutive heterochromatin results in somatic mosaicism in the expression of the euchromatic gene. This effect, known as position effect variegation, results from the functional inactivation of the euchromatic gene. Position effect variegation has been described in a variety of plants and animals and has been extensively studied in drosophila (see reference 57 for a review). Many workers have observed a striking correlation between the proportion of cells in which the variegating gene is packaged into heterochromatin and the degree to which a gene is expressed (19, 42). At the genetic level specific mutations which modify position effect variegation in *D. melanogaster* have been described previously (43). The localization of the C1A9 antigen-encoding gene to 29A on the 2L chromosome is very intriguing. Recently, a group of genes referred to as suppressors of position effect variegation have been genetically mapped to approximately 28.9 centimorgans on the 2L chromosome (55). Mutations at this locus result in marked supression of position effect variegation. It was suggested that genes at such loci may encode structural NHC protein components of heterochromatic regions. The protein we identified is an NHC protein and is very specifically associated with heterochromatic regions. The chromosomal location of the gene that encodes this protein at map position 29A appears to be at or very near to the mutation at 28.9 centimorgans. Preliminary studies indicate that the expression of the message for the C1A9 antigen is affected by this suppressor mutation (T. C. James and J. C. Eissenberg, unpublished data). It may therefore be possible to rescue the lethal homozygous phenotype by introducing an intact wild-type gene coding for the C1A9 antigen into this mutant by P element-mediated transformation (45). Such experiments are currently being undertaken in this laboratory.

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