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Isolation and distribution of a *Drosophila* protein preferentially associated with inactive regions of the genome

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Abstract. The distribution patterns of chromosomal proteins from *Drosophila* can be observed by immunofluorescent staining of the polytene chromosomes from larval salivary glands. We have purified a non-histone chromosomal protein of $M_r = 69000$ molecular weight which has a high affinity for DNA with little sequence specificity. Immunofluorescent staining indicates that this protein is preferentially associated with the inactive portions of the genome, including the centric heterochromatin and the condensed bands within the euchromatic arms of the chromosomes. Observation of both the heat shock loci 87A and 87C and the developmentally regulated loci 74EF and 75B shows an inverse correlation between immunofluorescent staining for the $M_r = 69000$ protein and for RNA polymerase. The presence of this protein appears to be correlated with the packaging of the chromatin in an inactive form.

Introduction

The DNA of eucaryotes is associated with histones and non-histone chromosomal proteins (NHC proteins) in a complex structure referred to as chromatin. Changes in certain general features of this structure are critical in gene activation and inactivation, as seen by correlated changes in the nuclease sensitivity of a locus (Weintraub and Groudine 1976; Garel and Axel 1976). Visible changes to a more open structure occur in some cases, e.g. in insect polytene chromosome puffs and in amphibian lampbrush chromosome loops. In addition to these changes in higher order structure, histone-DNA interactions are altered in the activation process as well (e.g. Wu et al. 1979b; Cartwright and Elgin 1986).

The NHC protein fraction is present at 30% to 120% compared with the mass of DNA (Elgin and Weintraub 1975); several hundred NHC proteins can be identified by two-dimensional gel electrophoresis (Peterson and McConkey 1976). Most of these proteins are not tissue specific, although there are cell specific qualitative and quantitative differences in the gel patterns. Many NHC proteins no doubt have a general function, some being involved in the general processes of gene activation and inactivation. A first step in elucidating the function of an NHC protein is to analyse its distribution within the chromatin. The polytene chromosomes of the *Drosophila* salivary gland from third instar larvae provide an excellent system with which to do this. These chromosomes can be easily observed under the light microscope and the distribution of a protein can be correlated with the well-defined genetic and molecular map. One can thereby obtain some information as to the biological processes in which a given protein might be involved. The distribution patterns of a number of well-characterized chromosomal proteins have been analysed using this approach, e.g., RNA polymerase II (Plagens et al. 1976; Jamrich et al. 1977a; Elgin et al. 1978), ribonucleoproteins (Christensen et al. 1981), DNA topoisomerase I (Fleischmann et al. 1984), and DNA topoisomerase II (Heller et al. 1986), as well as several of unknown function (Alfageme et al. 1976, 1980; Silver and Elgin 1977; Mayfield et al. 1978; Saumweber et al. 1980; James and Elgin 1986). Results to date indicate that some NHC proteins are associated with a large subfraction of the genome (i.e., heterochromatin), whereas others are associated with smaller subfractions (i.e., genes which are being actively transcribed).

In this paper we describe a simple procedure for the purification of a chromosomal protein of $M_r = 69000$ (P69) based on nuclear isolation, differential extraction with KSCN, differential precipitation with ammonium sulphate, and ion exchange chromatography. We describe the association of P69 with transcriptionally inactive regions of the genome, and compare it with the distribution of RNA polymerase II. The presence of this protein appears to be correlated with the packaging of the chromatin in an inactive form.

Materials and methods

Isolation of nuclei from Drosophila embryos. Nuclei were prepared from portions of 200 g of 6–18 h embryos of *D. melanogaster* Oregon R by a modification of the procedure of Hewisch and Burgoyne (1973) as described by Wu et al. (1979a), except that the final centrifugation was through 1.7 M sucrose. In an alternative procedure crude nuclei were prepared according to the method described by Javaherian et al. (1982). In both cases the nuclei were washed in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25 M sucrose, pelleted for 15 min at 10000 rpm in a Sorvall HB 4 rotor and stored frozen at −70° C.

Separation of proteins by CM-Sephadex C-25 chromatography. $(NH_4)_2SO_4$ precipitated protein samples were dialysed for 1 h against 7.5 mM sodium borate buffer, pH 8.8. The

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sample was spun in an Eppendorf Minifuge, and the supernatant loaded on a column containing 20 ml of CM-Sephadex C-25 equilibrated with the same buffer. Elution followed with a gradient of 0 to 1 M NaCl in 200 ml of borate buffer. Fractions of 1 ml were collected, diluted to at least 0.3 M NaCl, made 0.1 M HCl, and precipitated overnight at −20° C with 6 vol. of acetone. The NaCl concentration was determined by measuring the conductivity.

SDS polyacrylamide gel electrophoresis. Protein samples were analysed using SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970). The molecular weight of the proteins was determined in 10% to 30% polyacrylamide gradient gels using as molecular weight markers aldolase (160 kDa), *Escherichia coli* RNA polymerase (165 kDa, 155 kDa, 39 kDa), bovine serum albumin (BSA; 68 kDa), ovalbumin (43 kDa), and trypsin inhibitor (21.5 kDa). The gels were run at 10 V/cm for 2 days. Total embryo or nuclear extracts were prepared by homogenizing embryos or nuclei in an equal volume of sample buffer (Laemmli 1970). The sample was boiled for 3 min and any pellet removed by centrifugation for 10 min. Aliquots of the supernatant were loaded directly on the gel.

Extraction of proteins from polyacrylamide gels. Proteins were extracted from SDS-polyacrylamide gels after staining the gel with Coomassie blue. The desired band was excised, ground to small pieces and extracted several times at room temperature with electrophoresis buffer, until the gel pieces were totally destained. Proteins were precipitated by addition of 100% (w/v) trichloroacetic acid to a final concentration of 25% (w/v).

DNA binding of proteins. Protein-DNA binding was investigated with a modified nitrocellulose filter binding assay based on that originally developed by Riggs et al. (1970). A nitrocellulose disk of 5 mm in diameter was incubated with 0.5 µg of protein in 50 µl of buffer A [10 mM Tris-HCl, pH 7.6, 0.1 mM disodium EDTA, 1 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT), 0.1 mg/ml BSA, 60 mM KCl] for 3 h at room temperature. Incubation was continued overnight at 0° C with 3% BSA in 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1 mM phenylmethylsulphonylfluoride (PMSF) to block the remaining nitrocellulose binding sites. The nitrocellulose-bound proteins were washed twice in buffer B (same as buffer A but 1 mg/ml BSA and various concentrations of KCl) and immediately incubated with ^{32}P-endlabelled restriction fragments of DNA for 2 h at room temperature in 50 µl of buffer B, followed by extensive washing in the same buffer. Bound restriction fragments were eluted with 10 mM Tris-HCl, pH 7.6, 1 mM disodium EDTA, 0.1% SDS and 50 µg/ml of yeast RNA and separated on an agarose gel to estimate the relative amounts.

Preparation and use of the antiserum. Purified protein was run on a 10% SDS-polyacrylamide gel; the gel band representing the protein was excised, ground to small pieces and freeze dried. Three mice were immunized with these gel pieces by the procedure of Tjian et al. (1974) with minor modifications (Silver and Elgin 1976). Each injection contained 10 µg of protein. The sera of all three mice gave the same results. This holds true also for affinity purified anti-P69 antibodies (Fleischmann et al. 1984) from rabbit. In contrast no preimmune sera showed any fluorescent staining of the chromosomes over background level. The antiserum was used in the indirect immunofluorescence staining assay developed by Silver and Elgin (1976), using acetic acid and formaldehyde fixed chromosomes. Double immunofluorescent staining was done with mouse anti-P 69 serum and rabbit anti-RNA polymerase II serum. "Texas Red" conjugated goat anti-mouse IgG antibodies and fluorescein conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies. The specificity of each antiserum was shown by transferring the proteins from an SDS-polyacrylamide gel to nitrocellulose paper and incubating the gel replica first with the antiserum and subsequently with ^{125}I-labelled sheep anti-mouse F(ab′)$_2$ fragments (Towbin et al. 1979). An antiserum (P-125) against the large subunit of *Drosophila* RNA polymerase II was generously supplied by A. Greenleaf (Weeks et al. 1982).

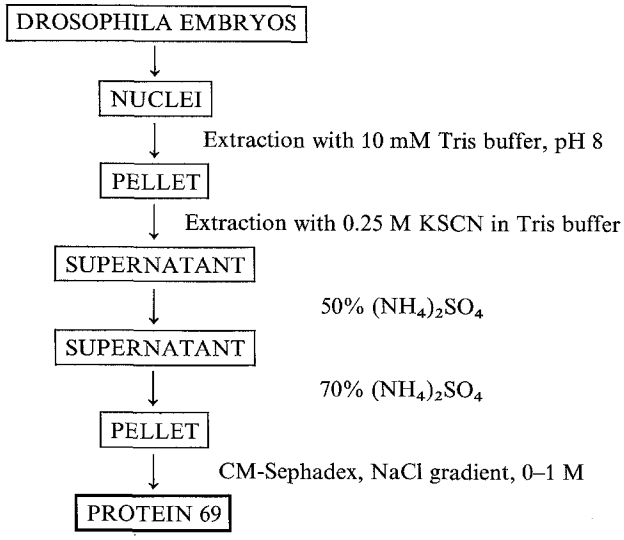

Fig. 1. Purification scheme for P69. Intermediate products for final purification are boxed with the purification steps written in between

Results

The purification of P69 is based primarily on differential extraction from nuclei and differential precipitation of the extracted proteins as outlined in Figure 1. A typical preparation starts with 2.4 kg of 6–18 h embryos and yields 30 g nuclei. The nuclei are first extracted twice by homogenization in 60 ml of 10 mM Tris-HCl, pH 8, 1 mM disodium EDTA, 1 mM 2-mercaptoethanol, 0.25 M sucrose. Each time centrifugation for 45 min at 330000 *g* follows. The pellet is again extracted twice in 40 ml of the same buffer containing 0.25 M KSCN as a chaotropic reagent, and pelleted as before. The supernatant contains P69; the pellet contains DNA associated with the histones, minor amounts of NHC proteins and only traces of P69. The supernatant protein is precipitated overnight by addition of solid $(NH_4)_2SO_4$ to 50% saturation. The proteins are spun down in a Sorvall HB4 rotor at 10000 rpm for 15 min. Next the $(NH_4)_2SO_4$ concentration of the supernatant is raised to 70% saturation. The resulting precipitate contains P69 (Fig. 3, lane C), but includes other proteins in this molecular weight range as well. This fraction is separated by CM-Sephadex C-25 chromatography as described in Materials

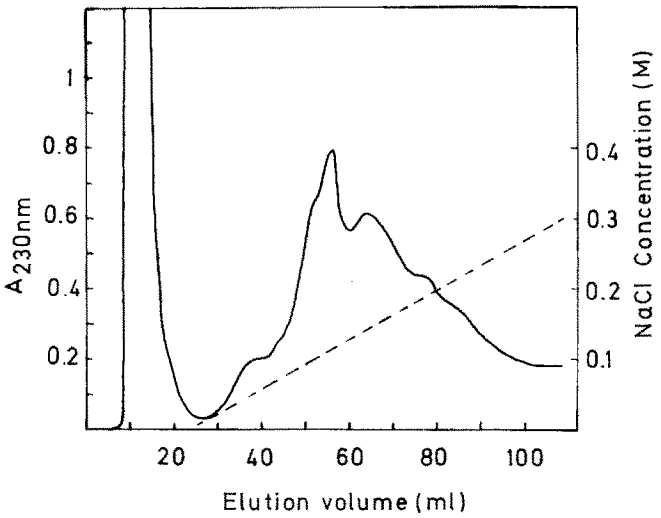

Fig. 2. Elution profile of P69 from CM-Sephadex C-25. The $(NH_4)_2SO_4$ precipitated protein sample was dissolved in 7.5 mM sodium borate buffer, pH 8.8, and chromatographed on a CM-Sephadex C-25 column equilibrated with the same buffer. Elution was by a gradient of 0–1 M NaCl. Fractions 60 to 72 (0.12–0.18 M NaCl) were pooled and acetone precipitated

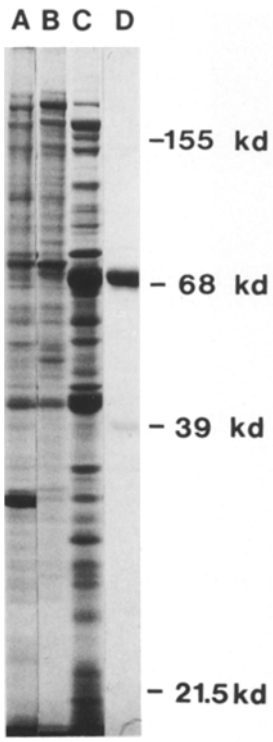

Fig. 3. Non-histone chromosomal (NHC) protein samples after successive purification steps for P69. Proteins were electrophoresed on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie blue. *Lane A* total nuclear proteins extracted with SDS gel sample buffer; *B* precipitate from 50% saturation with $(NH_4)_2SO_4$ of a 0.25 M KSCN extract of nuclei; *C* precipitate from 70% saturation with $(NH_4)_2SO_4$ of the supernatant of above; *D* pooled fractions 60 to 72 after elution from CM-Sephadex C-25. Almost pure P69 is seen in lane *D*. The $M_r = 69000$ protein band in lane C contains other proteins of the same molecular weight

Table 1. Purification of P69 from *Drosophila* embryos

Purification step	Material kept for further purification	Material discarded
6–18 h embryos	1 kg	
Preparation of nuclei	12.5 g	
10 mM Tris buffer extract of proteins		166 mg
0.25 KSCN extract of proteins	103 mg	
50% $(NH_4)_2SO_4$ precipitate of proteins		99 mg
70% $(NH_4)_2SO_4$ precipitate of proteins	4.0 mg	
Proteins bound to CM-Sephadex C-25	1.7 mg	
Proteins recovered from fractions 60–72	0.35 mg	
Yield of purified P69	0.25 mg[a]	

[a] Yield of P69 was estimated by the staining of the protein band in an SDS-polyacrylamide gel relative to a BSA standard. Approximately 0.1 mg of P69 is found in adjacent fractions

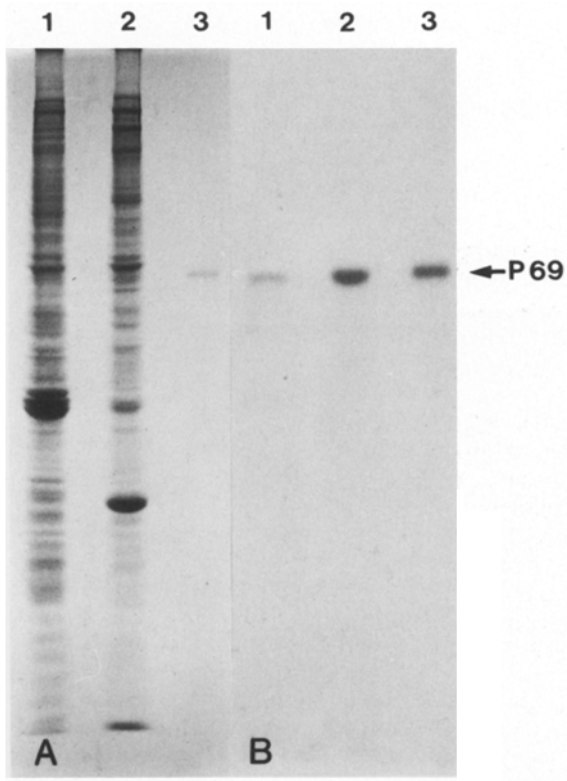

Fig. 4A, B. Selectivity of mouse anti-P69 serum. Proteins were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and successively incubated with anti-P69 serum and sheep anti-mouse Ig ^{125}I-labelled $F(ab')_2$ fragments and autoradiographed. **A** Coomassie blue stained gel, **B** autoradiograph. *Lane 1* total embryo proteins extracted with SDS gel sample buffer; *2* total nuclear proteins extracted with SDS gel sample buffer; *3* purified and gel extracted P69

and methods. The fractions containing 120 to 180 mM NaCl are pooled (fractions 60 to 72 of Fig. 2). The pooled proteins are separated on an SDS-polyacrylamide gel into one main band and a few minor bands as shown in Figure 3, lane D. The M_r of the major band was determined on SDS-polyacrylamide gels to be 69000. The yield of P69 is approximately 0.25 mg per 1 kg of embryos. Final purification is achieved by extraction of P69 from the SDS-polyacrylamide gel slice.

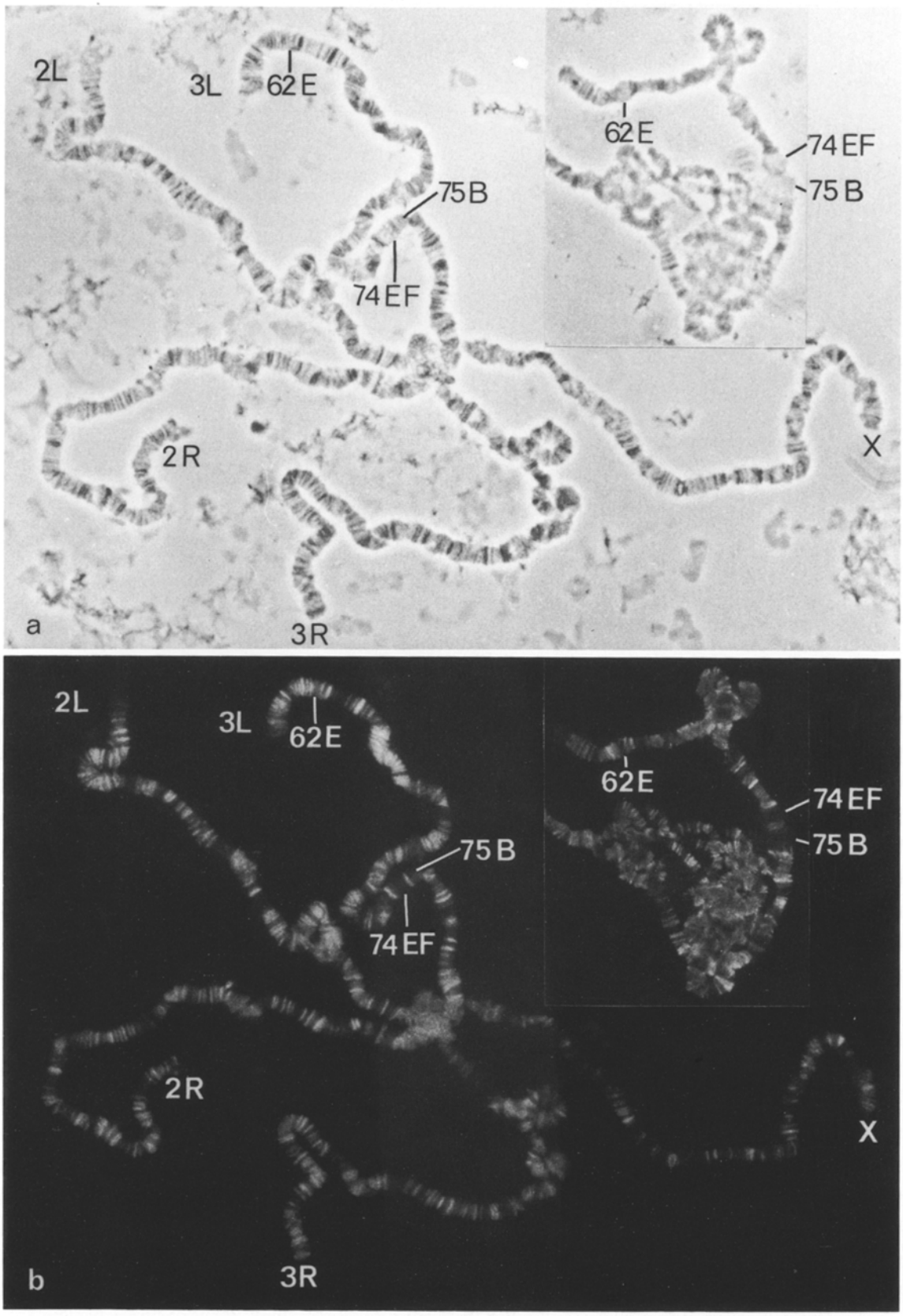

Fig. 5a, b. Distribution pattern of P69 on the polytene chromosomes. Chromosomes were prepared from salivary glands of third instar larvae fixed in 45% acetic acid and 3.7% formaldehyde. The chromosome arms and the developmental puffs 74EF, 75B, and 62E are indicated. The *insert* shows chromosome arm *3L* at a slightly later stage in development. **a** phase contrast; **b** fluorescence

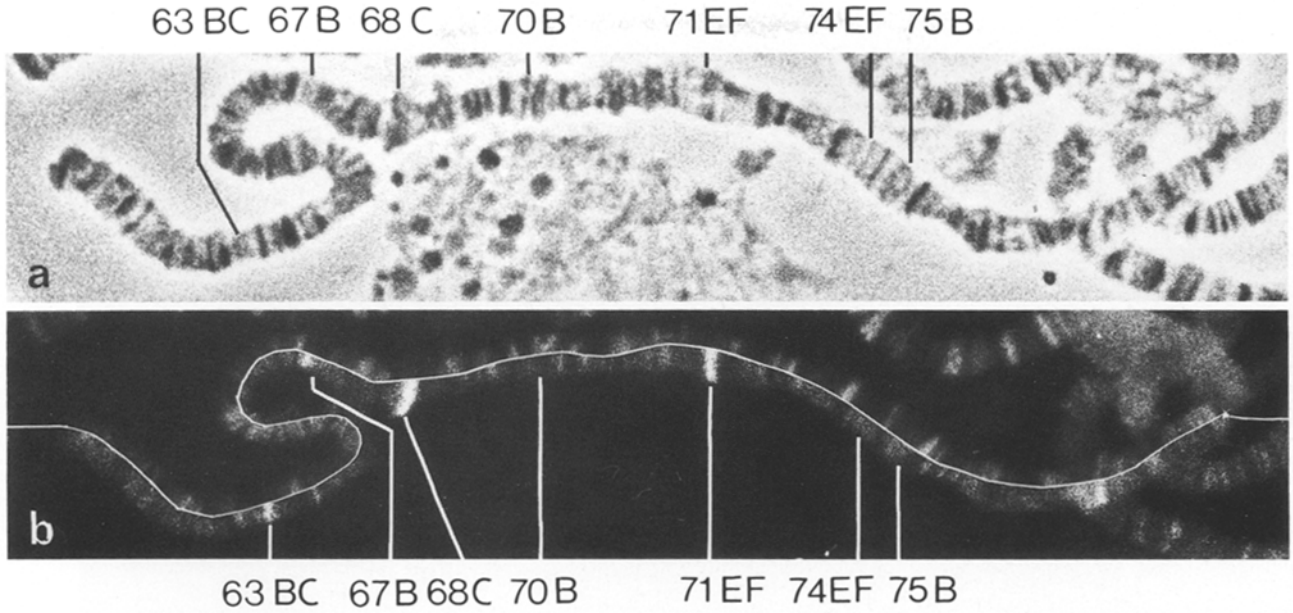

Fig. 6a, b. Distribution pattern of P69 and RNA polymerase II on polytene chromosome arm *3L*. **a** phase contrast, **b** fluorescence, showing in the upper part the distribution pattern of P69 and in the lower part the distribution pattern of RNA polymerase II

In an alternative procedure we used a crude nuclear preparation (Javaherian et al. 1982) to obtain P69. This procedure gives a higher yield of nuclei and up to 1 mg of P69 from 1 kg of embryos, but the protein is not as pure. A further disadvantage is the degradation of some of the proteins. Table 1 shows the recovery of material for each purification step. It is not possible to calculate the enrichment of P69 exactly because the initial amount could not be determined. Since we could isolate from crude nuclei of 1 kg of embryos 70 to 90 mg of histone H1, we estimate that there is on average 1 molecule of P69 per 250 molecules of H1. From the size of the H1 band in Figure 4A, lane 2, and the radioactive signal of P69 in Figure 4B, lane 3, we estimate that there is 1 molecule of P69 per 50 molecules of H1. From these estimates, the maximal yield of purified P69 is 20%. The enriched protein is stable for several months at −20° C in solution or lyophilized.

An antiserum against P69 was prepared; the specificity of the serum is demonstrated in Figure 4. The samples analysed included a crude embryo extract, a crude nuclear extract (see Materials and methods) and purified P69. These were run on an SDS-polyacrylamide gel, the proteins transferred to nitrocellulose and the nitrocellulose replica incubated with the antiserum. In all three cases the antibodies reacted almost exclusively with the $M_r = 69000$ band (Fig. 4). Note that P69 occurs in the embryo extract at a significantly lower concentration than in the nuclear extract, indicating a nuclear association for P69.

Incubation of polytene chromosome squashes with the antiserum results in a distinctive chromosome staining pattern (Fig. 5). Neither cytoplasm nor nucleoplasm shows fluorescence above the level observed by staining with the secondary antibody alone. P69 shows a marked association with the phase-dark bands and with the centric heterochromatin. The staining is complementary to that of RNA polymerase II, as shown clearly by a double-staining experiment using different primary and secondary antibodies (Fig. 6). No staining is observed in puffs or nucleoli (see also Fig. 7). Loci that puff as part of the developmental programme appear to be unstained or understained even prior to the development of a visible puff. A relatively low level of general staining is observed using chromosomes from salivary glands pre-fixed with formaldehyde, suggesting that the antigen is obscured or denatured by this procedure (data not shown). To analyse further the specific association of P69, chromosomes were prepared from third instar larvae heat shocked for 15 min at 37° C immediately prior to use. Under these conditions a limited set of genes is switched on and can be seen as the so-called heat shock puffs (Ritossa 1962; Ashburner 1972; Tissieres et al. 1974), while the expression of the developmentally active genes is severely reduced (McKenzie et al. 1975; Spradling et al. 1975). After heat shock there is a considerable reduction in staining for P69 in the region of the major heat shock puffs 87A and 87C, while there is now a high level of staining for RNA polymerase II at these loci (Fig. 7). There is no indication of a redistribution of P69 to the now repressed developmentally regulated puffs.

As P69 is a chromosomal protein, a high affinity for binding to DNA can be suggested. DNA binding was analysed with a nitrocellulose filter binding assay (based on that of Riggs et al. 1970). Table 2 shows that binding of P69 to DNA of *Drosophila* at increasing salt concentrations is not markedly different from binding to the *E. coli* plasmid vector DNA. P69 does not discriminate between coding and noncoding regions of the *Drosophila* yolk protein genes (data not shown), indicating a general binding to DNA with little sequence specificity.

Discussion

The purification procedure described in this paper represents a relatively simple technique for the preparation of substantial amounts of protein P69. An important step in purification of this protein is the isolation of the nuclei. P69 in a total *Drosophila* embryo extract is only stable for a few days even at −20° C. A nuclear extract can be stored for several months at this temperature (data not

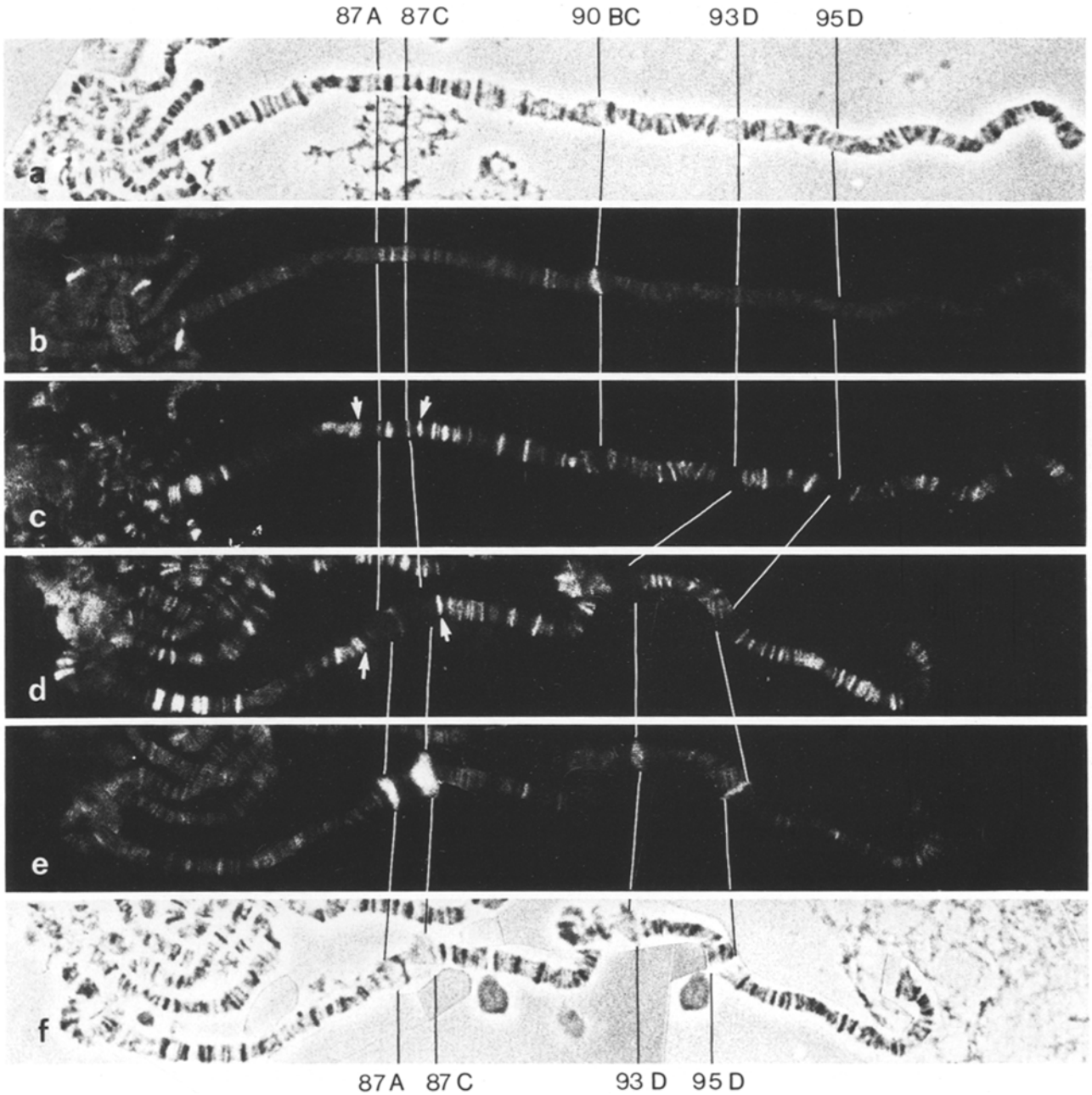

Fig. 7a–f. Distribution pattern of P69 and RNA polymerase II on polytene chromosome arm 3R from heat-shocked and non-heat-shocked animals. Chromosomes were obtained from third instar larvae grown at 25° C (**a, b, c**) and from larvae heat shocked at 37° C for 15 min prior to dissection (**d, e, f**). **c, d** Distribution pattern of P69, **b, e** distribution pattern of RNA polymerase II. Chromosomes in **d, e, f** were stretched by photo montage. Artefact next to locus 95BC (**d, e, f**) is caused by a short part of a crossing chromosome. The *arrows* indicate the same bands on both chromosomes. **a, f** phase contrast; **b, c, d, e** fluorescence

shown). An 80-fold reduction in material is achieved by this step. Further important purification steps are the differential extraction with KSCN and the differential precipitation with $(NH_4)_2SO_4$. These simple steps give a 120-fold and a 25-fold enrichment. The total amount of P69 present in nuclei can be estimated to be no more than 15000 molecules per nucleus. This purification scheme can be used as the basis for obtaining many other chromosomal proteins. Chromosomal proteins more tightly bound to nuclear components can be extracted from the residual nuclei with 1–2 M KSCN (e.g., James and Elgin 1986).

That P69 is a nuclear protein is shown by the antibody reaction with total protein extracts from embryos and nuclei. It is not extracted from nuclei at low ionic strength (10 mM Tris-HCl), but can be extracted with 0.25 M KSCN. The nuclear origin of this protein has been confirmed by the immunofluorescent images of its distribution on the polytene chromosomes and by studies of its binding to DNA. P69 appears to bind generally to DNA, though further tests of this idea are needed. Therefore the selective association with different loci observed in the polytene chromosomes may be mediated by other components of the chromatin. Note that P69 is not associated exclusively with polytene chromosomes; it was isolated from diploid

Table 2. Binding of P69 to DNA at different KCl concentrations

KCl (mM)	A	B
60	2746 (2409)	66 (83)
80	2610 (2452)	64 (78)
100	2140 (2361)	57 (82)
120	1590 (1684)	66 (84)
140	944 (1693)	63 (92)
160	662 (1732)	64 (97)
180	514 (763)	73 (98)
200	322 (517)	68 (98.5)

Proteins were bound by a modified procedure based on that of Riggs et al. (1970) to an equimolar mixture of a 2700 bp fragment of the pUC8 vector of *Escherichia coli* and a 1660 bp fragment containing the 1221 bp intergenic region of the *Drosophila* yolk genes 1 and 2 (Hovemann and Galler 1982). Both fragments were labelled at the 3′ ends with [^{32}P]dATP. Almost no DNA was bound to nitrocellulose in control experiments (63 to 96 cpm). A, protein-bound DNA in cpm; B, bound *Drosophila* DNA expressed as a percentage of A. In parentheses, DNA bound to a still uncharacterized *Drosophila* protein to demonstrate the reliability of the experiment

nuclei of *Drosophila* embryos, and the antibodies stain other nuclei in the salivary gland squashes. The polytene chromosomes are used because they can be easily observed under the light microscope, providing excellent resolution in a map of the whole genome. They behave like diploid interphase chromatin in many assays of function and fine structure (Cohen and Gotchel 1971; Tissieres et al. 1974; Elgin and Boyd 1975; Woodcock et al. 1976; Bonner and Pardue 1976; Hill et al. 1982).

P69 is associated with the compact regions of centric heterochromatin as well as with many condensed regions along the chromosome arms. Note, however, that the pattern does not show a simple correlation with mass. P69 is not found in transcriptionally active sites such as puffs or nucleoli. In fact, RNA polymerase II and P69 give almost inverse patterns (Fig. 6). In a few cases both proteins appear to be associated with the same locus, e.g. in 87C in the non-heat-shocked sample (Fig. 7). This may represent a transitional state, as some stress is generated during the preparation of the chromosomes, potentially leading to a low level of gene activation. Whether or not this is indeed the case must be resolved by techniques capable of higher resolution. After induction of the heat shock loci, P69 cannot be found in the large puffs at 87A and 87C. The protein is not observed either before or after induction at the other heat shock loci. Most of these genes are expressed in some tissues as part of a programme of developmentally regulated transcription (Ireland et al. 1982; Cheney and Shearn 1983; Zimmerman et al. 1983; Sirotkin and Davidson 1982). P69 does not appear in the heat-shock-repressed, developmentally regulated puffs. These results indicate that P69 is probably not a protein which reversibly represses gene activity to a low level, but one that functions in association with regions permanently inactivated in this tissue, perhaps playing a role in compaction into a higher-order structure. There seems to be close to an inverse correlation between association of P69 and association of the RNA polymerase with the chromosomes. Studies using UV-photocrosslinking techniques (Gilmour and Lis 1985) might be able to confirm and extend these results. Further biochemical and genetic analysis of this chromosomal protein is certainly warranted.

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