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Heat-shock puff 93 D from *Drosophila melanogaster*: accumulation of a RNP-specific antigen associated with giant particles of possible storage function

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The monoclonal antibody P11 is directed against a 38 000 dalton protein of *Drosophila melanogaster*. On polytene chromosomes this protein is present in a subset of the RNA polymerase II-containing loci. Here we show by density centrifugation and enzyme-linked immunosorbent assay tests that the P11 antigen is part of nuclear ribonucleoprotein (RNP) complexes. Indirect immunofluorescence shows that, after prolonged heat-shock, the P11 antigen is present only in the heat-shock puff 93 D. Identical distribution patterns were obtained with another monoclonal antibody, Q18. Unlike P11, this antibody also cross-reacts with *D. hydei* and *D. virilis* polytene chromosomes, where the puffs 48 B and 20 CD, respectively, are the only loci prominently stained after heat-shock. The small and giant RNP complexes previously described in these puffs were also observed in puff 93 D. Both types of particle contain the P11 antigen as shown by immunoelectron microscopy. We suggest that the P11 antigen is associated with a special class of RNPs which are possibly involved in the storage of primary transcription products inside the nucleus.

Key words: heat-shock/puffing/*D. melanogaster*/ribonucleoprotein/giant particles

Introduction

In an attempt to dissect the proteins associated with active chromatin, Saunweber *et al.* (1980) raised a series of monoclonal antibodies against chromosomal proteins of *Drosophila melanogaster*. The antigens corresponding to some of these antibodies (called class 1 antibodies) were localized by immunofluorescence in puffs and interbands of polytene chromosomes. A direct comparison of these immunofluorescence patterns with those obtained by antibodies directed against RNA polymerase II showed that none correctly overlaps with the pattern of transcriptional activity (Kabisch and Bautz, 1983).

To correlate the class 1 antibody fluorescence patterns with gene activation and inactivation in an experimentally defined system, we took advantage of the heat-shock response in *Drosophila*. Heat-shock of larvae leads to the immediate formation of a limited number of puffs with high transcriptional activity. On the other hand, it causes a pronounced reduction of transcriptional activity at non-heat-shock loci (reviewed by Ashburner and Bonner, 1979). We therefore used polytene chromosome squashes from heat-shock larvae to screen for fluorescence in the easily discernible heat-shock puffs and observed that a group of antibodies (P11, Q14, Q16 and Q18, henceforth called the P11 group) decorate only one chromosomal locus after heat-shock, namely the heat-shock puff 93 D (Dangli and Bautz, 1983).

Antibodies P11 and Q16 have been found, by immunoblotting, to be directed against a 38 000 dalton polypeptide and antibody Q18 against a 55 000 dalton polypeptide (Saumweber *et al.*, 1980; Risau *et al.*, 1983). Here we show by density gradient analysis and the enzyme-linked immunosorbent assay (ELISA) test that the P11 antigen is a ribonucleoprotein (RNP)-associated protein and we describe: (i) the fluorescence pattern of P11 in 93 D after heat-shock, (ii) the submicroscopic structure of 93 D, (iii) the localization of the P11 antigen in particles which specifically accumulate in 93 D following heat-shock by immunoelectron microscopy, and (iv) the Q18 fluorescence pattern on *D. hydei* and *D. virilis* polytene chromosomes following heat-shock.

Results

The P11 antigen is associated with nuclear RNP fractions

RNA and proteins of *D. melanogaster* tissue culture cells were labelled with 5-[^{3}H]uridine and [^{35}S]methionine, respectively, for 1 h. The heterogeneous nuclear RNP (hnRNP) complexes were isolated from purified nuclear preparations and analyzed by cesium sulfate density gradient centrifugation. As shown in Figure 1a, the majority of the labelled hnRNA bands at a buoyant density of between $\varrho = 1.27$ g/cm^3 and $\varrho = 1.32$ g/cm^3, which corresponds to an RNA:protein ratio of ~1:4 for the isolated hnRNP complexes. Small amounts of labelled RNA banding at a density position of $\varrho = 1.52$ g/cm^3 probably represent stripped hnRNP complexes. To see whether P11 antigen is associated with the isolated RNPs, we tested aliquots of the gradient fractions for the presence of P11 antigen with the monoclonal P11 antibody by ELISA (Figure 1b). The results show that the P11 antigen is almost exclusively localized in the hnRNP fractions of the cesium sulfate gradient. Furthermore, preliminary data using u.v. cross-linking suggest that the P11 antigen is not homogeneously distributed among the hnRNP complexes as it cannot be found in certain distinct RNP fractions (Kloetzel and Bautz, in preparation).

P11 antigen in 93 D

Following heat-shock, the P11 antigen could be localized by indirect immunofluorescence almost exclusively in the heat-shock puff 93 D (Dangli and Bautz, 1983). As shown in Figure 2a, the P11 staining in this puff is not homogeneous but appears rather granular. Fluorescent granules can also be observed in the nucleoplasm of incompletely squashed salivary gland cells following heat-shock (Figure 2b).

Submicroscopic morphology of heat-shock puff 93 D and localization of the P11 antigen

The localization of P11 antigen in 93 D after heat-shock and the granular appearance of the P11 staining are accompanied by exceptionally large RNP structures in this puff, as shown by sections through glutaraldehyde-fixed and squashed chromosomes (Figure 3a, b).

In non-heat-shock chromosomes only a few of these giant RNP particles were visible over the band 93 D 6-7 (Figure 3a). In this band the heat-shock gene was recently mapped by

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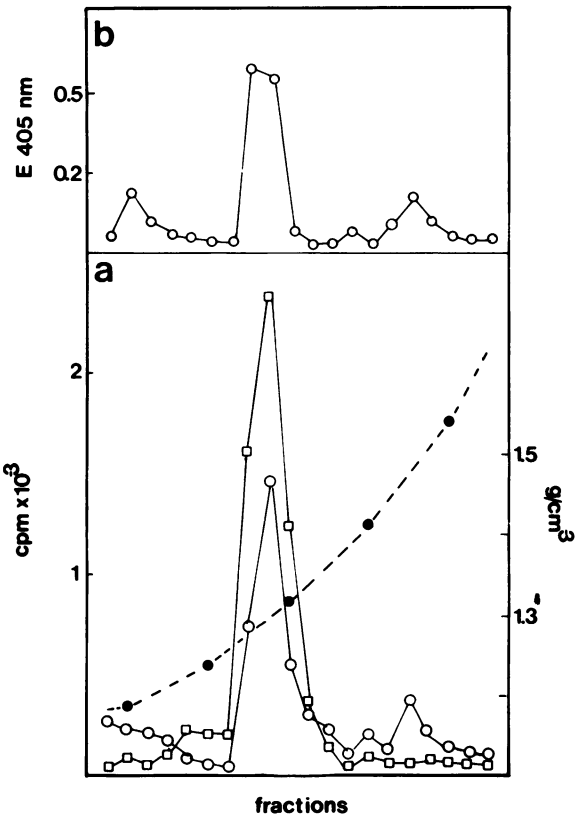

Fig. 1. Cs_2SO_4 density gradient analysis of [^{3}H]uridine and [^{35}S]methionine labelled hnRNP of *D. melanogaster* tissue culture cells and analysis of the distribution of the P11 antigen using the P11 antibody in ELISA. **(a)** Distribution of ^{3}H-labelled RNA (○——○) and ^{35}S-labelled protein (□——□). **(b)** Amount of P11 antigen present in the individual gradient fractions as measured by the OD at 405 nm after ELISA.

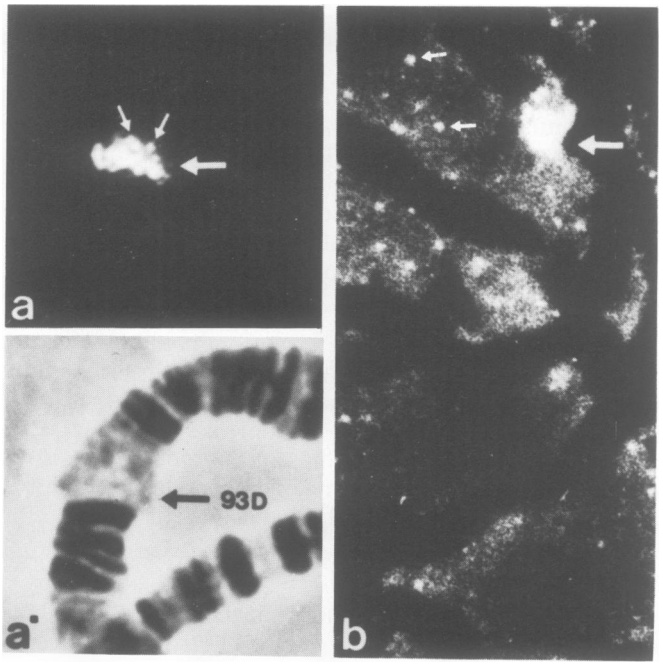

Fig. 2. Brightly fluorescent granules obtained by P11 in 93 D (in **a**) and in the nucleus of incompletely squashed salivary gland cells (in **b**). **(a)** and **(b)** Indirect immunofluorescence micrographs. Puff 93 D is indicated by a large, granules by small arrows. **(a')** Phase contrast view of the chromosome shown in **a**. Larvae were heat-shocked for 60 min at 37°C.

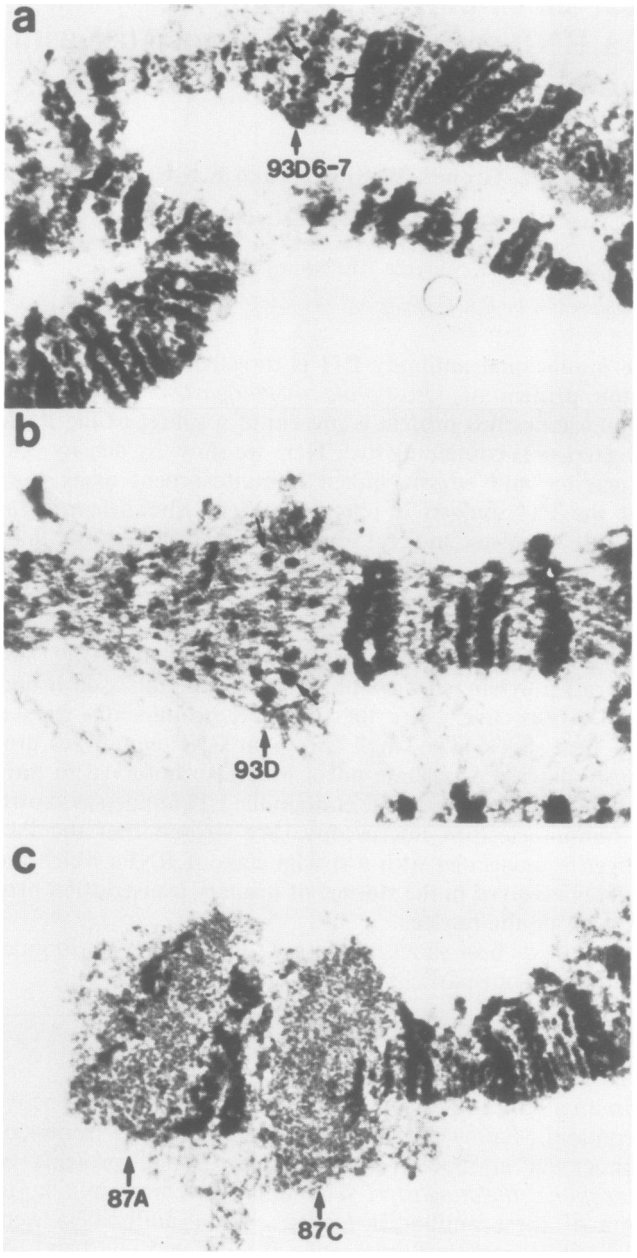

Fig. 3. Giant RNP granules in 93 D. Electron micrographs from sections through glutaraldehyde-fixed, squashed chromosomes. **(a)** 93 D region at 25°C. Giant particles over the band 93 D 6–7 are indicated by arrows. **(b)** 93 D region following heat-shock (60 min at 37°C). Giant particles are indicated by arrows. **(c)** Puffs 87 C and 87 A following heat-shock (60 min at 37°C). No giant particles are visible. (Magnification: 8900 x.)

deletion analysis (Mohler and Pardue, 1982). The number of giant particles in 93 D increases after puff induction by heat-shock (Figure 3b). Similar particles cannot be detected in other chromosomal loci such as, for example, the heat-shock puffs 87 A and 87 C (Figure 3c). In sections of salivary gland nuclei these particles can be found in heat-shock puff 93 D as well as free in the nucleoplasm. No particles are seen outside the nucleus. These non-squashed preparations permitted a detailed study of the 93 D and the free nucleoplasmic particles. They are roughly spherical, ~300 nm in diameter, and small particles on stalks protrude from their irregular surface (Figure 4b). Besides these giant complexes, small 30 nm par-

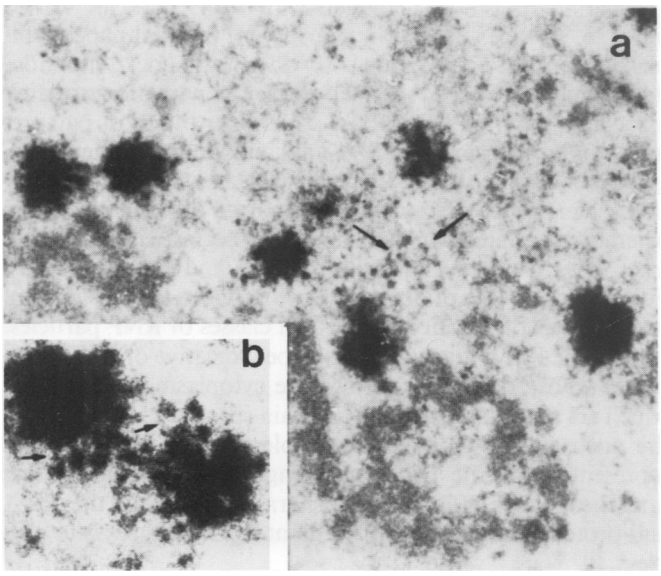

Fig. 4. Fine structure of 93 D and the giant particles. Electron micrograph of a section through a whole gland following heat-shock (60 min at 37°C). Glands were fixed in glutaraldehyde and OsO_4 and stained in uranyl acetate. (**a**) Detail of puff 93 D. Besides the giant particles, small particles are also visible (arrows). (Magnification: 25 000 x.) (**b**) Fine structure of nucleoplasmic giant particles: small granules are connected by stalks (arrow) to the core of the particle. (Magnification: 52 000 x.)

ticles are also seen in the 93 D puff (Figure 4a). The characteristic ultrastructure of 93 D thus resembles the previously described ultrastructure of the heat-shock puffs 48 B in *D. hydei* and 20 CD in *D. virilis* (Derksen *et al.*, 1973; Swift, 1965).

The granular appearance of the P11 fluorescence observed in 93 D suggested that the P11 antigen is associated with the giant particles present in this puff. For submicroscopic localization of the P11 antigen in 93 D, we used an indirect immunoperoxidase method on glutaraldehyde-fixed and squashed chromosomes which allows visualization of the antigen on both light and electron microscopic levels. In the light microscope, the immunoperoxidase stain was confined to puff 93 D after 60 min heat-shock (Figure 5a). The P11-stained chromosome sites could thus be selected under the light microscope and subsequently sectioned for electron microscopic analysis. As shown in Figure 5b and c, the P11 antigen was found to be associated with both the giant and the small RNP granules in puff 93 D.

P11 group antigen in the puffs 48 B of D. hydei and 20 CD of D. virilis

The observation that 93 D shares a common ultrastructure with the puffs 48 B of *D. hydei* and 20 CD of *D. virilis* provides additional evidence for the earlier suggestion (see Discussion) that these loci are equivalent. This conclusion is further supported by the finding that 48 B and 20 CD are the only chromosomal loci of *D. hydei* and *D. virilis*, respectively, which are prominently stained after prolonged heat-shock by Q 18 (Figure 6), a P11 group antibody which, in contrast to P11, cross-reacts with *D. hydei* and *D. virilis* chromosomes (Saumweber *et al.*, 1980).

Discussion

Here we show by cesium sulfate gradient analysis and the ELISA test that the P11 antigen is part of nuclear hnRNP

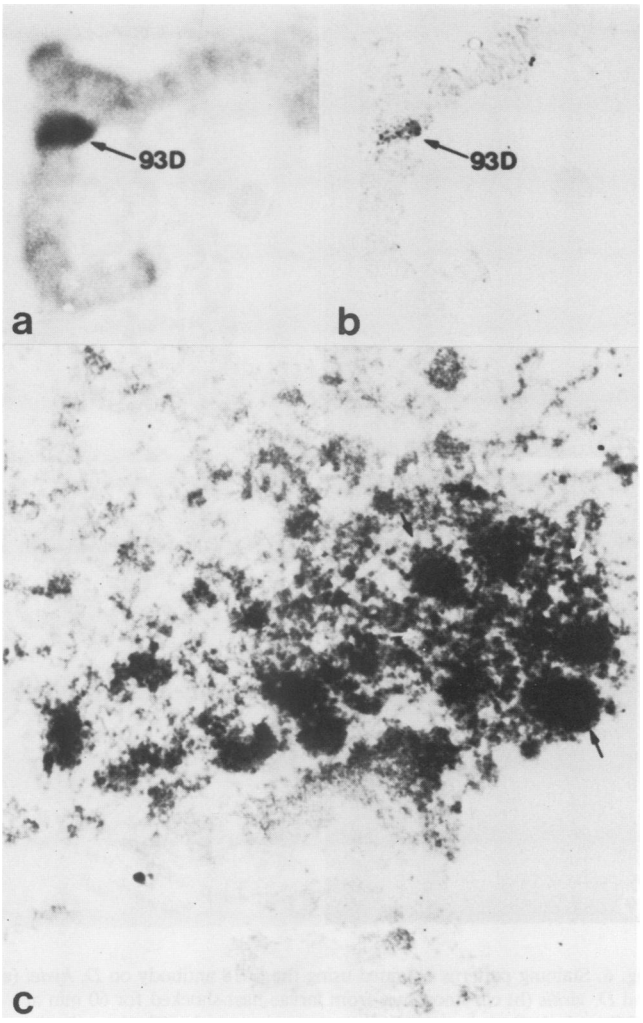

Fig. 5. Localization of the P11 antigen in 93 D. Larvae were heat-shocked (60 min at 37°C). Chromosomes were glutaraldehyde fixed and squashed. P11 antigen was visualized by immunoperoxidase staining. (**a**) Light microscopic and (**b**) electron microscopic view of the same chromosome. (Magnification: 1750 x.) (**c**) Detail of the 93 D puff shown in **b**. Staining can be observed on the giant (black arrows) as well as on the small (white arrows) particles. (Magnification: 25 000 x.)

complexes. This is in agreement with data showing that the P11 antigen is present in RNase-sensitive sucrose gradient fractions (Risau *et al.*, 1983). These data were supplemented by the recent finding that, upon u.v. cross-linking, the P11 antigen is in direct contact with particular fractions of nuclear RNA (Koetzel and Bautz, in preparation). Under heat-shock conditions, the P11 antigen is found cytologically in the 93 D puff associated with 30 nm particles as well as with the giant RNP structures characteristic of this locus. This finding indicates that components of the small particles are also present in the giant RNPs. Such a relationship between the 30 nm and the giant particles was also suggested in the case of heat-shock puff 48 B from *D. hydei* on the basis of cytochemical data (Derksen and Willart, 1976).

The observation that the Q 18 antibody decorates a single heat-shock puff in *D. melanogaster* (93 D), *D. virilis* (20 CD) and in *D. hydei* (48 B), as well as the ultrastructural similarity of these puffs, suggest that they are functionally equivalent. This is also supported by earlier data: all three puffs can be induced independently of the other heat-shock puffs (Lakhotia

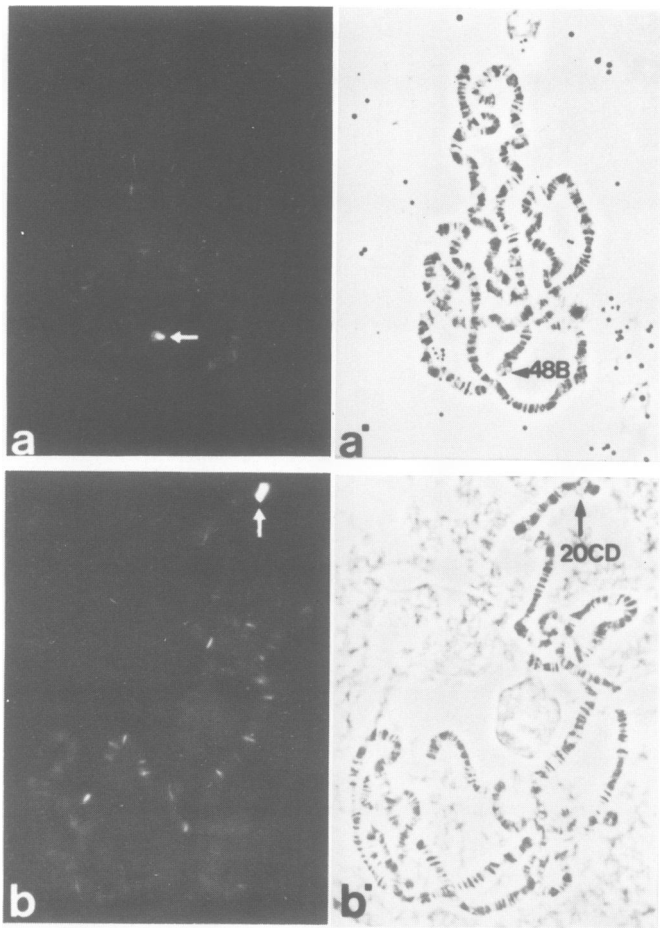

Fig. 6. Staining patterns obtained using the Q 18 antibody on *D. hydei* (**a**) and *D. virilis* (**b**) chromosomes from larvae heat-shocked for 60 min at 37°C. **a, b**: indirect immunofluorescence micrographs. The heat-shock puffs 48 B of *D. hydei* and 20 CD of *D. virilis* are indicated by arrows. **a', b'**: phase contrast view of the same chromosomes.

and Mukherjee, 1970, 1980; Leenders *et al.*, 1973; Bonner and Pardue, 1976; Gubenko and Barisheva, 1979; Lakhotia and Singh, 1982); 93 D and 48 B map close to the *ebony* locus (D'Alessandro *et al.*, 1977; Henikoff, 1980; Grond *et al.*, 1982) and produce a RNA that remains largely in the nucleus and is unlikely to code for a protein (Lengyel *et al.*, 1980; Lubsen *et al.*, 1978; Peters *et al.*, 1982). These similarities of the described loci are, however, not paralleled by conservation at the level of primary DNA structure (Peters *et al.*, 1980, 1982). Interestingly, the protein recognized by the P11 antibody also appears to have changed during evolution, as indicated by the lack of cross-reactivity of the P11 antibody (in addition to Q14 and Q16 which also belong to the P11 group) with *D. hydei* and *D. virilis* chromosomes (Saumweber *et al.*, 1980).

The P11 antigen is present in many sites transcribed by RNA polymerase II though not in all (Kabisch and Bautz, 1983). Little if any P11 antigen is, for example, associated with genes coding for salivary glue proteins. The primary transcription products of these genes are supposed to be quickly transported into the cytoplasm, where they are translated efficiently. On the other hand, puffs described to contain RNA which remains in the nucleus for a prolonged period were found to possess high levels of P11 antigen. On the basis of this evidence the authors suggested a specific association of the P11 antigen with RNP particles with a possible storage function. As shown in this paper, this reasoning can be extended to the heat-shock situation. Following heat-shock, the P11 antigen is part of a structure associated with the 93 D RNA which, for the most part, remains in the nucleus. The P11 antigen is, on the other hand, apparently not associated with the RNAs of the other heat-shock loci, which are quickly transported into the cytoplasm whereby translation of these messages begins within minutes after onset of heat-shock (Ashburner and Bonner, 1979). Following this line of argument, we propose the existence of several types of functionally distinct classes of RNP particles. Hence, the P11 antigen appears to be indicative of RNP complexes whose transposition into the cytoplasm is delayed or which act as storage particles to retain specific RNA species in the nucleus. The giant RNP complexes in 93 D might thus represent a specific form of storage particle accumulating after heat-shock. Biochemical characterization of the RNA and protein composition of the giant RNP particles is in progress.

Materials and methods

Cell culture

The *D. melanogaster* tissue culture cell line Kc 0 (Echalier and Ohanessian, 1970) was used. The cells were grown in suspension in D_{22} medium containing 2% fetal calf serum (FCS). The cells were grown in spinner flasks at 24°C and were adjusted to concentrations of between 2 x 10^6 and 6 x 10^6 cells/ml.

RNA and protein labelling conditions

Tissue culture cells were harvested by centrifugation at 1000 r.p.m. for 5 min. Cells were resuspended in D_{22} medium minus yeast extract and FCS and incubated for 1 h at 24°C with 5-fluorouridine (5 μg/ml) which selectively inhibits rRNA synthesis in cultured *Drosophila* cells (Lengyel and Penman, 1975). Cells were then collected again at 1000 r.p.m. for 5 min, resuspended in the same medium plus 5-fluorouridine at a 5- to 6-fold higher cell concentration and incubated at 24°C for 30 min for recovery before further handling. For RNA labelling, 15 μCi/ml 5-[^{3}H]uridine (sp. act. 40–60 Ci/mmol), for protein labelling 10 μCi/ml [^{35}S]methionine were added and the cells incubated for the desired length of time.

Cell fractionation and isolation of RNP

Cells were diluted in 100 mM NaCl, 10 mM $CaCl_2$, 100 mM Tris/HCl pH 7.2 and pelleted at 1000 *g* for 5 min. Cells were washed twice in the same solution and resuspended in 15 volumes of 30 mM NaCl, 10 mM $CaCl_2$, 100 mM Tris/HCl pH 8.5 and 0.5% Nonidet P-40 (NP-40). Cells were lysed for 10 min on ice and the nuclei were pelleted at 1000 *g* for 5 min. The nuclei were resuspended in 10 mm NaCl, 10 mM Tris/HCl pH 7.2, 1.5 Mg/Cl_2 (RSB) and layered on top of an 8% sucrose cushion made up in the same solution. Nuclei were pelleted at 1500 *g* for 10 min and the nuclear preparations were checked for purity under the microscope. For the isolation of hnRNP, nuclei were sonicated for 2 x 30 s in RSB plus 5 mM EDTA. The homogenate was layered on top of a 30% sucrose cushion in RSB and centrifuged at 6000 *g* for 20 min to remove insoluble material and most of the chromatin. The hnRNP on top of the cushion was used for further analyses.

Determination of RNP buoyant density

For density determination in Cs_2SO_4, RNP complexes were homogenized in RSB plus 0.2% NP-40 and analyzed on 20–55% preformed Cs_2SO_4 gradients as described previously (Kloetzel *et al.*, 1982).

ELISA test

For the identification of P11, antigen aliquots of the Cs_2SO_4 gradient fractions were applied to microtiter plates for antigen binding. Using P11 monoclonal antibody to identify P11 antigen, the alkaline phosphatase reaction was performed essentially as described by Voller *et al.* (1976).

Heat-shock treatment of larvae

Third instar *D. melanogaster* (Oregon R, *D. hydei* and *D. virilis* (wild-type) larvae, grown at room temperature, were used. The larvae were heat-shocked (60 min at 37°C) by transferring them into an Eppendorf tube which was immersed in a pre-warmed waterbath.

Indirect immunofluorescence

Indirect immunofluorescence was performed as described by Jamrich *et al.*

(1977) with the modifications of Saumweber *et al.* (1980). For preparing polytene chromosome squashes, salivary glands were dissected in phosphate buffered saline (PBS)/1% Triton-X-100 (pH 7.4). Fixation was in 3.7% formaldehyde in dissection medium (30 s) and in 45% acetic acid/3.7% formaldehyde (60 – 90 s). Squashes were performed in the latter solution. For the first antibody reaction, cell culture medium supernatants in a 1:5 dilution were used. The monoclonal antibodies P11 and Q 18 have been described by Saumweber *et al.* (1980). Fluorescein isothiocyanate-labelled anti-mouse IgG in a 1:40 dilution (Miles, Yeda) was used as a second antibody.

Preparation of salivary-gland chromosomes for electron microscopy

Chromosome squashes. Salivary glands were dissected in Ringer, fixed for 15 min on ice in a 3% glutaraldehyde solution in 0.1% cacodylate buffer pH 7.2 and subsequently for 15 min in fresh fixative at room temperature. Following fixation they were squashed in 45% acetic acid, frozen in liquid nitrogen and post-fixed after removal of the coverslip in methanol:37% formaldehyde (9:1).

Cytological analysis. The chromosomes were hydrated, stained with hematoxylin, ethanol dehydrated and stained with uranyl acetate, embedded in epon, marked and prepared from electron microscopy as described in detail by Derksen (1978).

Immunoperoxidase localization of antigens. The slides were hydrated in an ethanol:37% formaldehyde series (7:3, 5:5, 3:7, 10 min each), washed in PBS (twice for 10 min) and incubated with the antibodies as for immunofluorescence, with the exception that a peroxidase-conjugated anti-mouse antibody (Cappel Laboratories, Cochranville, USA) in a 1:10 dilution was used as the second antibody. After two 10 min washes in PBS and one in Tris-buffered saline (TBS), the preparations were incubated for 5 min in a freshly prepared medium containing 0.05% diaminobenzidine.4 HCl and 0.03%/H_2O_2 in 0.01 M Tris-Cl (pH 7.6). After washing in TBS twice for 10 min, the preparations were stained for 1 h in 1% OsO_4, dehydrated and prepared for electron microscopy as described above, except that the staining with hematoxylin and uranyl acetate was omitted.

Preparation of salivary glands for electron microscopy

Salivary glands were fixed overnight at 4°C in a 6% glutaraldehyde solution in 0.1 M cacodylate buffer and subsequently in fresh fixative for 30 min at room temperature. They were then washed extensively with the cacodylate buffer, post-fixed for 1 h in 2% OsO_4, dehydrated in an ethanol/water series and embedded in epon. Sections were stained with uranyl acetate. The method is described in detail by Derksen *et al.* (1973).

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