Identification of Histone H2b as a Heat-shock Protein in *Drosophila*

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ABSTRACT Total cell polypeptides synthesized, in cultured *Drosophila* cells under control (25°C) and heat-shock (37°C) conditions have been compared in two different two-dimensional polyacrylamide gel electrophoresis systems which, together, resolve polypeptides having a wide range of isoelectric points, including the most basic polypeptides of the cell. The electrophoresis of basic proteins showed that the most prominent basic polypeptide synthesized in heat shock comigrated with histone H2b. This heat-shock polypeptide was identified as histone H2b by two criteria: (*a*) it comigrated with authentic histone H2b in Triton-urea-acetic acid acrylamide gel electrophoresis after solubilization from nuclei with acid; and (*b*) partial proteolysis peptide maps of the basic heat-shock protein and histone H2b were identical. The synthesis of histone H2b was induced threefold in heat shock, whereas synthesis of the other histones was reduced from two- to tenfold. The noncoordinate synthesis of histones in *Drosophila* in heat shock provides an interesting system in which to investigate transcriptional and translational controls of histone synthesis as well as assembly of histones into chromatin.

The heat-shock response in *Drosophila* was first observed as a puffing response at nine specific loci on the polytene salivary chromosomes in larvae that were transferred from 25°C to 37°C (1). Subsequently, it has been found that the response involves the induction of synthesis of a number of RNAs, several of which code for a series of polypeptides synthesized during heat shock, the heat-shock proteins (2–6). The mechanism of induction and the physiological meaning of the response are not understood, and up to this time none of the heat-shock polypeptides has been identified.

I have characterized the heat-shock polypeptides in *Drosophila* by two-dimensional polyacrylamide gel electrophoresis (PAGE) of basic as well as neutral and acidic polypeptides from solubilized whole cells. These experiments have led to the identification of the smallest and most basic heat-shock protein as histone H2b.

MATERIALS AND METHODS

Cell Culture and Labeling Conditions

The A4E6 line of *Drosophila* Kc cells (7) was obtained from Peter and Lucy Cherbas, of Harvard University, and was maintained at 25°C in tissue culture flasks in D22 medium (8) supplemented with 10% heat-inactivated fetal calf serum (FCS) at densities between 1 and 10 × 10^6 cells/ml. For heat-shock experiments the cells were centrifuged, washed once with D22 minus methionine, and resuspended at a density of 1 × 10^7 cells/ml in D22 minus methionine containing 10% dialyzed FCS. The culture was divided in half and 100 μCi/ml [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) was added to each culture 20 min after the beginning of the incubation period at 25°C (control) or 37°C (heat shock). At the end of the incubation the cells were centrifuged and the medium was removed.

Two-Dimensional Gel Electrophoresis

Residual medium was removed from pellets of 1 × 10^7 cells by carefully blotting the pellet with a cotton-tipped applicator, and the cells were immediately dissolved in 100 μl of lysis buffer (9) or 100 μl of lysis buffer containing 0.3 M NaCl and 1.6 mg/ml protamine to displace basic proteins from nucleic acid (see footnote 4 in reference 10). The dissolved cells were transferred to a 1.5-ml capped microcentrifuge tube, sonicated for 30 s, and stored frozen (10, 11).

Electrophoresis of acidic and neutral proteins (11) and of basic proteins (10) was done as previously described, except that 12.5% acrylamide gels were used for the second dimension. A 20-μl aliquot was loaded on the first-dimension gel; control and heat-shocked samples were electrophoresed in pairs. The gels were stained with Coomassie brilliant blue R, destained, dried, and autoradiographed using Kodak XR-5 x-ray film.

Preparation of Nuclei and Histones

Pelleted cells from heat-shocked and control cultures were immediately resuspended in cold B buffer (60 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 0.001% spermidine, 0.01 M Tris, pH 7.4) containing 0.5% NP-40 (Nonidet) using 2 ml of B buffer per 10^8 cells. The cells were broken in a Dounce homogenizer with a tight-fitting pestle, and nuclei were pelleted by centrifugation at 9,100*g* for 10 min. For extraction of histones, the nuclei were resuspended in the above B buffer minus NP-40 and digested for 10 min at 25°C with 25 U/ml micrococcal nuclease in the presence of 1 mM $CaCl_2$. Digestion was stopped by the addition of NaCl to 2 M and HCl to 0.1 M. Histones were prepared for electrophoresis as previously described (12).

Subcellular Fractionation

Heat-shocked cells labeled with [^{35}S]methionine were homogenized as described above and centrifuged at 9,150*g* for 10 min. The supernatant fluid was removed and called "cytoplasm". The pelleted crude nuclear fraction was washed in B buffer plus 0.22 M NaCl (total NaCl plus KCl concentration of 0.3 M). Proteins in the 0.3 M salt nuclear wash and in the cytoplasm were precipitated by

the addition of 2 vol of 95% ethanol, left in the freezer overnight, and centrifuged (12). The 0.3 M salt-washed nuclear pellet and the two ethanol pellets were dissolved in loading buffer and subjected to discontinuous SDS-PAGE on 0.8-mm thick 12.5% gels (13).

Triton-Urea-Acetic Acid Gel Electrophoresis

Histones were electrophoresed on Triton-urea-acetic acid slab gels in the first dimension and discontinuous SDS gels in the second as described previously (12, 14), except that 0.37% Triton DF-16 and 8 M urea were used for the first dimension.

Peptide Mapping by Partial Proteolysis

Sample slots on a 0.8-mm thick, 18% acrylamide discontinuous SDS slab gel (13) were loaded with 5 μl of freshly prepared 100 μg/ml subtilisin Carlsberg in loading buffer (15) followed by 15 μl of loading buffer. The histone H2b spots were cut out of stained, dried two-dimensional basic protein gels and pushed into the sample slots (16). After a 20-min incubation at room temperature, electrophoresis was started at 5 mA, constant current. When the tracking dye reached the running gel, the current was increased to 15 mA.

RESULTS

The heat-shock proteins of *Drosophila* were characterized by two-dimensional PAGE of solubilized whole cells in two electrophoresis systems that together display polypeptides having a broad range of isoelectric points, including the most basic polypeptides of the cell. The neutral and acidic polypeptides labeled under control conditions at 25°C and during heat shock at 37°C were compared on two-dimensional isoelectric focusing/SDS gels (9, 11) (Fig. 1 *a* and *b*). The number and properties of the heat-shock polypeptides in Fig. 1 *b* are similar to the pattern previously reported by Mirault et al. (17). Seven

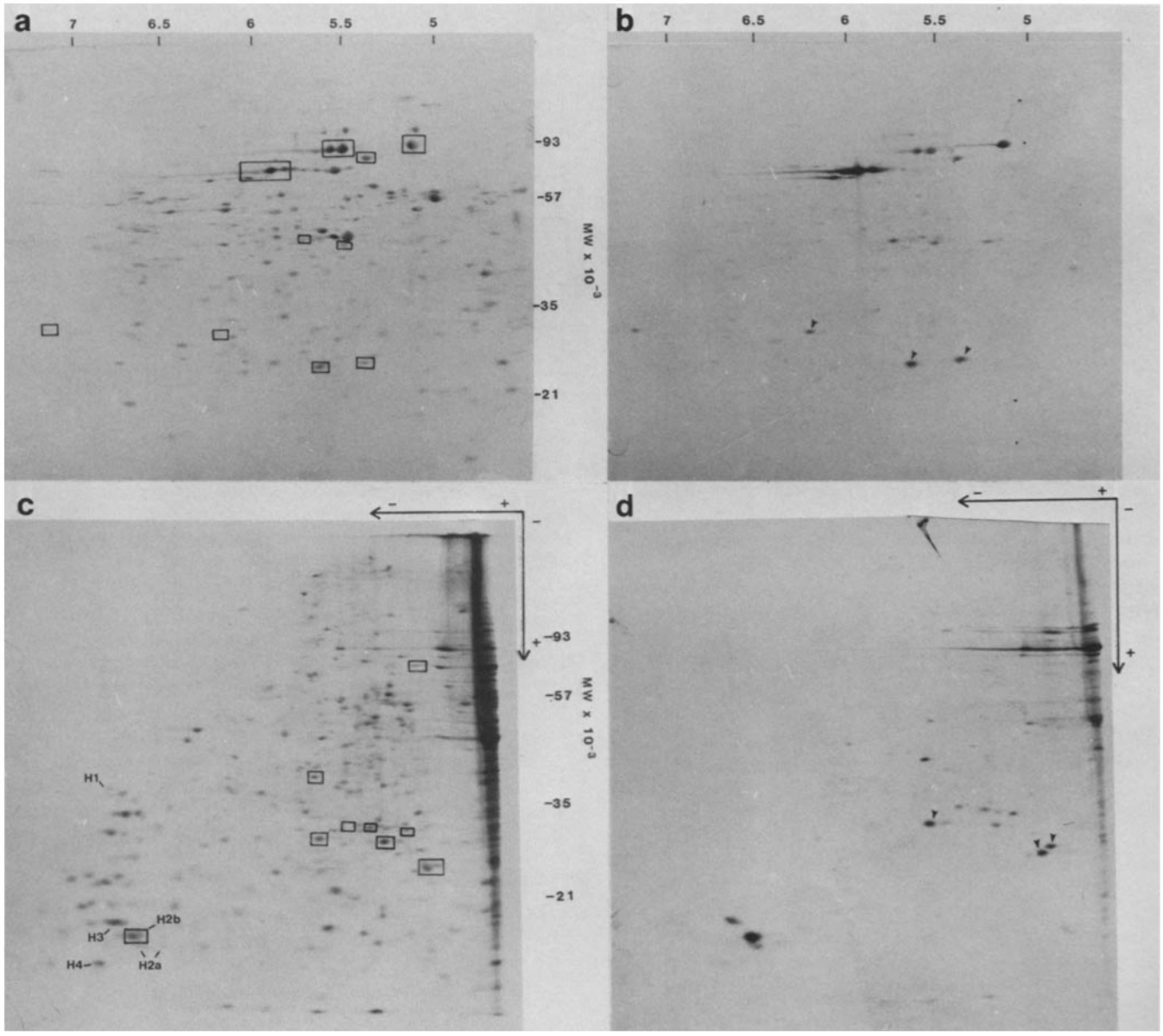

FIGURE 1 Autoradiogram of two-dimensional gels of solubilized *Drosophila* cells at 25° and 37°C. (*a* and *b*) Isoelectric focusing gels of control and heat-shocked cultures, respectively, pH measured in 0.5-cm segments of a first-dimension gel is indicated across the top of *a*. (*c* and *d*) Basic protein gels of control and heat-shocked cells, respectively. The histones are identified in *c*. Boxes in *a* and *c* show the position of migration of the comparable heat-shock polypeptide. The arrows in *b* and *d* identify lower molecular weight heat-shock polypeptides discussed in the text that are present on both the isoelectric focusing and basic protein gels.

or eight major and several minor heat-shock polypeptides are present. A careful comparison of the proteins synthesized at 25°C and 37°C demonstrates that all of the major heat-shock polypeptides incorporate label under control (25°C) conditions. These proteins are indicated by boxes on the control gel (Fig. 1 *a*). In some cases (e.g., the 80,000- and 83,000-dalton polypeptides) their net rates of synthesis at 25°C and 37°C are similar. In other cases the rate of synthesis of the heat-shock polypeptides at 25°C is lower. Visual inspection of the stained gels shows that the 70,000-, 80,000-, and 83,000-dalton heat-shock polypeptides represent major proteins in the cell at 25°C (photo not shown). The detectable synthesis of heat-shock polypeptides in cultures growing at 25°C is not surprising in view of Findly and Pederson's (18) finding that messenger RNA coding for the 70,000- and 26,000-dalton heat-shock polypeptides is present at significant levels in cells growing at 25°C.

The basic proteins labeled under control and heat-shock conditions were displayed in a nonequilibrium pH gel electrophoresis system (10) and are shown in Fig. 1 *c* and *d*. More than five major heat-shock polypeptides are resolved by this system. Although the pattern of migration of the heat-shock polypeptides in the charge dimension is rather different from that seen in the isoelectric focusing gels shown in Fig. 1 *b*, the heat-shock polypeptides indicated by the arrows in the basic protein gel in Fig. 1 *d* have precisely the same molecular weight as the arrow-marked heat-shock polypeptides in the isoelectric focusing gel in Fig. 1 *b*, suggesting that they are the same polypeptides. This has been confirmed for the smallest two by partial proteolysis peptide mapping (data not shown). Further, as indicated by the boxes in panel *c*, the basic heat-shock polypeptides are present in control cells at 25°C. Finally, panel *d* shows that the most prominent low-molecular-weight basic heat-shock polypeptide comigrates with histone H2b. Visual inspection of control and heat-shock autoradiograms suggested that synthesis of the H2b-like protein is induced in heat shock whereas synthesis of the other histones is reduced. Quantitative densitometry of paired autoradiograms from control and heat-shock cultures indicated that synthesis of the H2b-like protein is three- to fourfold induced in heat shock whereas synthesis of histones H3, H4, and H1 is reduced to various extents (Table I). Synthesis of histone H1 under heat-shock conditions is only barely detectable. It was not possible to determine the level of histone H2a synthesis in heat shock from these data.

TABLE I

Rates of Incorporation of Amino Acids into Histones at 25°C and 37°C

Histone	25°C*	37°C*	Relative rate of synthesis in heat shock
H2b (10 d)‡	339	Off scale	—
(3 d)	103	335	3.2
H3 (10 d)	281	148	0.6
H4 (10 d)	200	32	0.2
H2a	*ND*§	*ND*	—
H1 (10 d)	Off scale	130	—
(2 d)	395	20	<0.1

Autoradiograms from an experiment like that shown in Fig. 1 *c* and *d* were scanned on an integrating densitometer. The data for H2b, H3, and H4 are from the same pair of autoradiograms from [^{35}S]methionine-labeled cells. Histone H1 data were from a comparable experiment with [^{3}H]lysine-labeled cells. Areas under the peaks for heat-shocked and control samples were compared to obtain a relative rate of synthesis in heat shock.

* Data reported are integrated areas of the peaks in arbitrary numbers.

‡ Exposure times for the autoradiograms are in parentheses.

§ Not determined.

Two types of experiments demonstrated that heat-shock H2b ($H2b^{hs}$) was actually histone H2b. First, putative $H2b^{hs}$ was extracted from nuclei with histones, and its electrophoretic properties on Triton-urea-acetic acid acrylamide gels were compared with those of histone H2b. The results of a subcellular fractionation experiment are shown in Fig. 2. After 1 h at 37°C, a number of heat-shock polypeptides were concentrated in the crude nuclear fraction, confirming the results of Arrigo et al. (19). About half of the putative $H2b^{hs}$ was present in the nucleus and was not solubilized by 0.3 M NaCl. Because 0.3 M NaCl removes loosely and/or nonspecifically bound nonhistone proteins from chromatin, this result suggested that the putative $H2b^{hs}$ was specifically, rather than fortuitously, bound to some nuclear structure. Nuclei from [^{35}S]methionine-labeled heat-shocked and control cells were therefore extracted with 0.1 N HCl, and the acid-soluble proteins were electrophoresed in two dimensions with Triton-urea-acetic acid gels in the first dimension and discontinuous SDS gel electrophoresis in the second. Fig. 3 shows that the major acid-soluble nuclear protein synthesized during heat shock comigrates with histone H2b. These data also show that histone H2a is synthesized in heat shock at ~ 75% of control levels. With the exception of histone H2a, the relative incorporation of [^{35}S]methionine into the histones under heat-shock and control conditions in this experiment is similar to that in Fig. 1 *d* and Table I, although

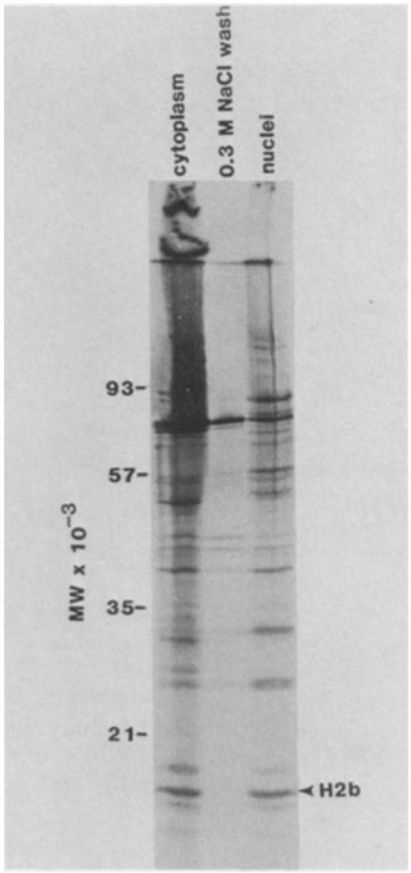

FIGURE 2 Subcellular fractionation of heat-shock polypeptides. The labeled proteins present in "cytoplasm," a 0.3 M NaCl nuclear wash, and 0.3 M NaCl-washed nuclei prepared as described in Materials and Methods were compared by electrophoresis on a 12.5% discontinuous SDS slab gel. The position of migration of histone H2b and molecular weight markers are shown.

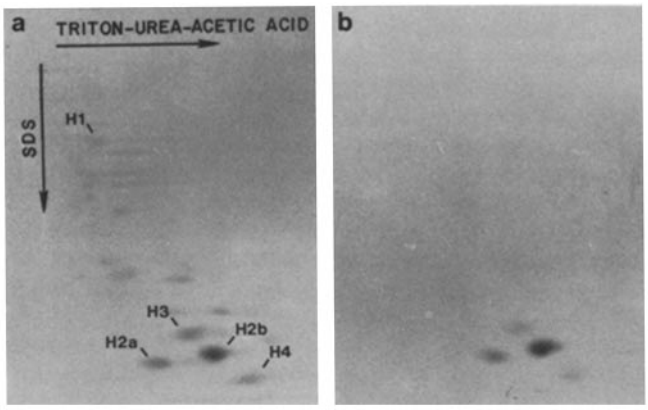

FIGURE 3 Two-dimensional electrophoresis of acid-soluble nuclear proteins from control and heat-shocked cells in Triton/urea/acetic acid and discontinuous SDS gels. Acid-soluble nuclear proteins were prepared from [^{35}S]methionine-labeled control and heat-shocked cultures and electrophoresed as described in Materials and Methods. (*a*) Control and (*b*) heat shock. The [^{35}S]methionine-labeled histones comigrated with the stained spots in all cases. The histones are identified in *a*.

only the proteins present in the nucleus are represented here. Thus, the nuclear complement of newly synthesized histones reflects the noncoordinate rates of total histone synthesis under heat-shock conditions.

In a second approach to identifying the most prominent basic heat-shock polypeptide in *Drosophila*, peptide maps of putative histone H2b^{hs} and authentic H2b were prepared by partial proteolysis under denaturing conditions (15). Histone H2b spots were located in the staining pattern of two-dimensional gels of basic proteins from solubilized [^{35}S]methionine-labeled control and heat-shocked cells. The spots were cut out, digested with subtilisin Carlsberg, and the digestion products were compared by discontinuous SDS-PAGE. Fig. 4 shows that the peptides from H2b labeled under control and heat-shock conditions were indistinguishable. These data and the coelectrophoresis of putative H2b^{hs} with authentic histone H2b shown in Fig. 3 identify the smallest and most prominent basic heat-shock polypeptide in *Drosophila* as histone H2b.

DISCUSSION

The experiments reported here demonstrate that synthesis of histone H2b is increased in heat shock in *Drosophila* cultured cells, whereas synthesis of histones H1, H3, H4, and H2a is reduced. Histone H2b is the first heat-shock polypeptide to be identified. Histone H2b is synthesized in cells growing at 25°C, as are other proteins induced in heat shock (Fig. 1 *a–d*). Synthesis at 25°C was especially striking for the unidentified 70,000-, 80,000-, and 83,000-dalton heat-shock polypeptides in addition to histone H2b. In these cases the presence of major stained spots in the gels rules out the possibility of induction of synthesis in control cells by the simple manipulations of the cells required to set up the experiments. The evidence for synthesis of heat-shock polypeptides at 25°C reported here corroborates the findings of Findly and Pederson (18) who showed that messenger RNAs coding for the 70,000- and 26,000-dalton heat-shock polypeptides are present in cells growing at 25°C. In view of these data, I propose that heat-shock polypeptides may be appropriately defined as polypeptides whose synthesis in heat shock is either induced or continues at the former rate. The most important implication of these findings is that, in heat shock, the cell makes increased amounts of normally present polypeptide to accommodate to the stress of heat shock rather than inducing new polypeptides for functions not required under normal growth conditions.

Electrophoresis of solubilized heat-shocked cells in a two-dimensional system for basic proteins resolved more than five prominent polypeptides whose synthesis was increased under heat-shock conditions (Fig. 1 *d*). These, when added to the neutral and acidic heat-shock polypeptides (Fig. 1 *b*), would at first appear to increase the number of prominent heat-shock polypeptides from ~8 (2) to as many as 12. (Definition of a minor heat-shock polypeptide is, obviously, somewhat arbitrary.) However, the polypeptides marked by arrows in Fig. 1 *b* and *d* appear in both gel systems. Determination of further overlap between the two gel systems, and thus a more accurate total count of heat-shock polypeptides, will require partial proteolysis peptide mapping of the other basic heat-shock polypeptides. It is clear, however, that the heat-shock histone appears only on the basic protein gel system. This observation increases the number of heat-shock polypeptides by at least one, to a minimum of nine.

The nucleosome histones (H2a, H2b, H3, and H4) are known to be present in chromatin in a molar ratio of 1:1:1:1 (20); histone H1 is present in roughly half that amount (21). The histone genes in *Drosophila* have been shown to be arranged in clusters, each of which contains one gene for each of the five histones (22); the relative rate of synthesis of the individual histone proteins is thought to be tightly regulated (22). The accumulation of [^{35}S]methionine into histones H2b, H3, and H4 in 40 min at 25°C (Table I) is consistent with a coordinate control of histone synthesis. At 37°C, however, histone H2b incorporated [^{35}S]methionine at three to four times the control rate whereas synthesis of histones H3, H4, and H1 is decreased by as much as tenfold. Thus, in heat shock, histone H2b accumulates fifteen times more rapidly than histone H4 and five times more rapidly than H3. The rate of synthesis of H2a is lower than that of H2b, but it cannot be quantitated at the present time. The finding that histone synthesis is noncoordinate in heat shock in *Drosophila* represents the first evidence that relative rates of histone synthesis can be perturbed. This observation raises a number of interesting questions concerning the regulation of histone synthesis, the fate of the histones synthesized in heat shock, and the consequences of noncoor-

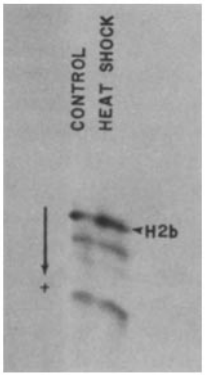

FIGURE 4 Peptide maps of authentic histone H2b and H2b^{hs}. Partial proteolysis of authentic [^{35}S]methionine-labeled H2b and H2b^{hs} by Subtilisin Carlsberg was carried out as described in Materials and Methods. The digestion products were electrophoresed on an 18% discontinuous SDS slab gel (13). The position of migration of undigested H2b is marked.

dinate histone synthesis for the cell.

Analysis of the subcellular distribution of histone $H2b^{hs}$ and other heat-shock polypeptides (Fig. 2) showed that a substantial fraction of the lower-molecular-weight heat-shock polypeptides are present in the cell nucleus, confirming the results of Arrigo et al. (18). Fig. 2 also showed that $H2b^{hs}$ and other nuclear heat-shock polypeptides were not dissociated by washing nuclei with 0.3 M NaCl. This observation suggests that $H2b^{hs}$ may be chromatin-bound; the nature of the association with chromatin is not clear, however. Turnover of histones in chromatin has been reported in another system (23); however, the present case may differ in that histone synthesis is clearly noncoordinate in heat shock in *Drosophila*.

Peptide mapping of $H2b^{hs}$ by partial proteolysis and coelectrophoresis of the $H2b^{hs}$ with normal histone H2b on Triton-urea-acetic acid gel electrophoresis were used to identify the most basic heat-shock protein as H2b. However, neither approach distinguished whether $H2b^{hs}$ is an amino acid sequence variant differing in only a few residues from the normal histone. Although such variants have not been described in *Drosophila* they occur in a number of other organisms (for review see reference 24). If $H2b^{hs}$ were an amino acid sequence variant, it would, by definition, be synthesized from a messenger RNA encoded by a structural gene different than that which codes for normal H2b in these cells.

The experiments reported here do not address the question whether induction of histone H2b in heat shock is regulated at the transcriptional or the translational level. The continued synthesis of histone messenger RNA during heat shock has been demonstrated by in situ hybridization of heat-shock messenger RNA to the histone locus in polytene chromosomes (5, 6). In addition, individual histone messenger RNAs have been purified from polysomes in heat-shocked cultured cells (25); however, the relative amounts of the individual histone messages were not determined. Experiments in progress are designed to elucidate whether the H2b induction in heat shock is transcriptionally or translationally regulated.

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