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Teratogens induce a subset of small heat shock proteins in *Drosophila* primary embryonic cell cultures

(muscle and neuron differentiation/β-ecdysterone/dose–response)

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ABSTRACT **_Drosophila_ embryonic cells placed into culture just after gastrulation differentiate _in vitro_ over the next 24 hr. A number of drugs that are teratogenic in mammalian systems have been found to inhibit muscle or neuron differentiation (or both) in these developing cultures. We have examined, by two-dimensional gel electrophoresis, the effects of these drugs on protein synthesis in embryonic cells. For nine teratogens tested, cells treated for 20 hr with the drug show a dramatic induction of three proteins of about 20 kilodaltons, in addition to the normal proteins synthesized by untreated cells. Three teratogens as well as all eight nonteratogens tested did not show this induction. The induced proteins appear to be identical to three of the heat shock proteins (hsp 23, 22a, and 22b), as shown by electrophoretic mobilities and peptide mapping by partial proteolysis. A 37°C heat shock of the embryonic cells produces the full complement of heat shock proteins, whereas drug-treated cells induce only the subset hsp 23, 22a, and 22b but not hsp 26 or 27. β-Ecdysterone, the _Drosophila_ molting hormone, also inhibits embryonic differentiation and induces hsp 23, 22a, and 22b, a partial subset of the heat shock proteins (hsp 22, 23, 26, and 27) induced by the hormone in imaginal discs and some _Drosophila_ continuous cell lines. Dose–response studies of several drugs show a correlation between the degree of inhibition of differentiation and the level of induction of hsp 23, 22a, and 22b. The induction of heat shock proteins by drugs may reflect specific types of stress that can also give rise to teratogenesis.**

The heat shock response in *Drosophila* has been extensively studied (for reviews, see refs. 1 and 2). A heat shock given to *Drosophila* at most stages of development results in a rapid initiation of transcription of a group of specific heat shock genes (3–6). The concomitant synthesis of a group of about seven major heat shock proteins is induced, while normal protein synthesis is repressed (3, 7). Two-dimensional gel electrophoresis has shown multiple isoelectric point variants of many of the major heat shock proteins (6, 8, 9). Among the small heat shock proteins, hsp 26 and 27 together consist of about eight isoelectric point variants, hsp 23 is a single component, and hsp 22 consists of two variant species (8). Four genes coding for hsp 22, 23, 26, and 27 have been cloned and localized to the 67B region of *Drosophila* salivary chromosomes (10–12).

Primary cell cultures prepared from *Drosophila* gastrulation stage embryos undergo differentiation *in vitro* during the next 24 hr (13). Among a number of cell types in these cultures, the differentiation of two has been studied in some detail. Myoblasts divide to yield myocytes, which elongate, align, and fuse to form myotubes (14). Neuroblasts undergo a series of divisions yielding clusters of neurons that send out axons and form miniature ganglia with a neuropile at the center and cell bodies at the periphery (15). Neuromuscular junctions are formed, resulting in spontaneous movements of the myotubes (16).

A number of drugs that are teratogenic in mammalian systems have recently been found to inhibit muscle or neuron differentiation (or both) in *Drosophila* primary embryonic cells (17, 18). In this investigation we examined protein synthesis in these cells treated with various drugs. We show that most drugs that inhibit differentiation in the primary cultures also induce the synthesis of three proteins that we have identified as hsp 23, 22a, and 22b. A preliminary account of this work has recently been presented (19).

METHODS

Preparation of Cultures and Radiolabeling. Mass primary cultures were prepared from *Drosophila* gastrulation stage embryos (Oregon R strain) at 5×10^6 cells per 35-mm dish, as described (20). After cells had attached (20–30 min), medium was removed and replaced with medium containing the drug to be tested. Cells were incubated at 25°C for 20 hr and then labeled in the presence of the drug for 1 hr with 200 μCi of [^{35}S]methionine (New England Nuclear; specific activity, ≈1000 Ci/mmol; 1 Ci = 37 GBq) per ml.

Heat treatments were given to primary embryonic cells at 20 hr after plating when muscle and neuron differentiation was essentially completed. Cells were incubated for 1 hr at 30°C or 37°C and labeled with 200 μCi of [^{35}S]methionine per ml during the entire heat treatment.

After labeling, the drug- or heat-treated cells were washed and solubilized by methods described previously (20).

Two-Dimensional Gel Electrophoresis and Analysis. Samples were separated by two-dimensional gel electrophoresis (21), using a pH 5–7 or pH 6–8 gradient (8) for isoelectric focusing and a 9–15% gradient acrylamide slab for NaDodSO$_4$/polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue, fluorographed (22), dried, and exposed to Kodak XAR film.

Integrated optical densities of proteins 22a and 22b were measured from fluorograms on a Bausch and Lomb automated image analysis system. Densities (in the linear region of the film) from drug-treated cells were normalized to the density of actin II from the same sample and expressed as relative increases over normalized control values. The levels of proteins 22a and 22b were about equal and have been averaged. Protein 23 was not well enough separated from protein N to obtain accurate measurements.

***Drosophila* Differentiation Assay.** Drug-treated or untreated control cultures plated at 1.6×10^6 cells per dish were allowed to differentiate at 25°C for 20 hr. Muscle and neuron differentiation was assayed by staining and counting myotubes and neuron clusters using a Bausch and Lomb automated image analysis system (18). Results were expressed as a percentage of the number of myotubes and neuron clusters in control cultures prepared on the same day.

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Abbreviation: hsp, heat shock protein(s).

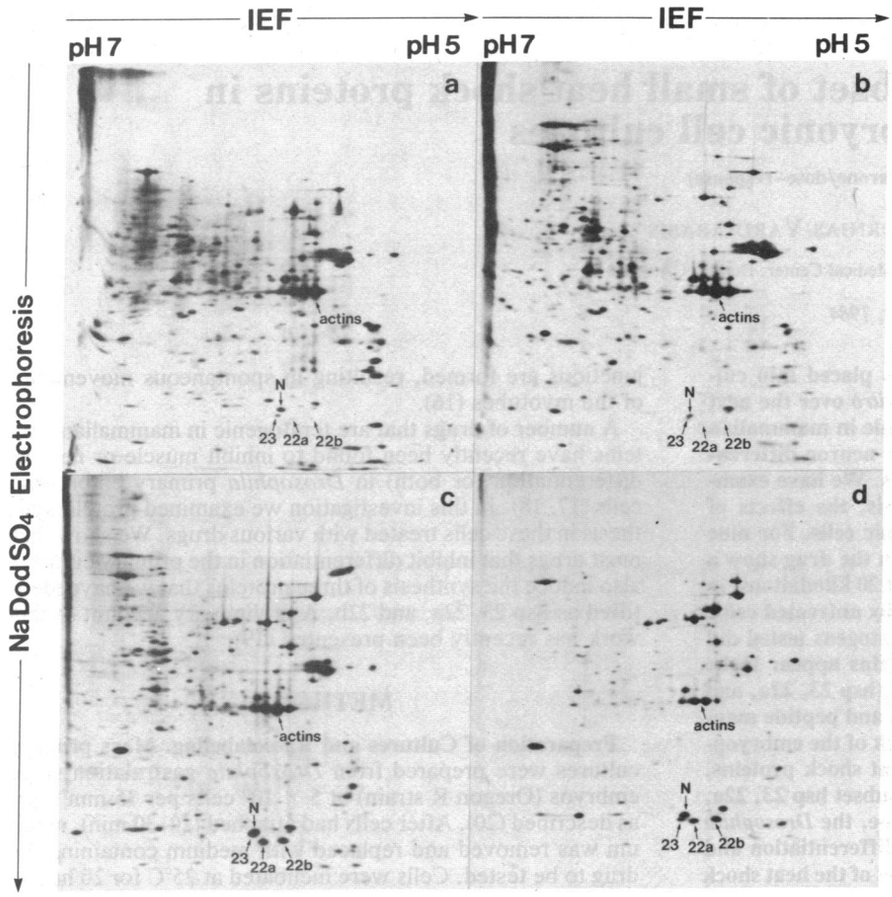

FIG. 1. Protein synthesis in drug-treated primary embryonic cells. *Drosophila* primary embryonic cells were plated just after gastrulation (3.5 hr after oviposition) at 5×10^6 cells per dish. The indicated drug was added at about 4 hr after oviposition and the cells were allowed to differentiate at 25°C for 20 hr in the presence of the drug. Cells were then labeled for 1 hr with [^{35}S]methionine (200 μCi/ml) in the presence of the drug. Cells were solubilized and proteins were separated by two-dimensional gel electrophoresis and visualized by fluorography. Drugs used and exposure times (cpm × days) were control (919,000) (*a*); cortisone, 0.01 mM (594,000) (*b*); diphenylhydantoin, 0.1 mM (424,000) (*c*); and coumarin, 1 mM (362,000) (*d*). N is a non-heat shock protein that migrates close to protein 23 (8). IEF, isoelectric focusing.

One-Dimensional Peptide Mapping. Proteolytic digestion and separation of peptide fragments were carried out according to Cleveland *et al.* (23). Proteins from heat-shocked or drug-treated cells were cut from two-dimensional gels, treated with 20 μg of *Staphylococcus aureus* V8 protease (Miles) per ml or 33 μg of chymotrypsin (Worthington) per ml, and analyzed electrophoretically. Separated peptide fragments were visualized by fluorography.

RESULTS

Drug-Induced Proteins. Muscle or neuron differentiation (or both) in *Drosophila* primary embryonic cells cultured *in vitro* can be inhibited by many drugs that act as teratogens in mammals. To determine the effects of these drugs on protein synthesis, we labeled embryonic cultures treated for 20 hr with either diphenylhydantoin (0.1 mM) or coumarin (1

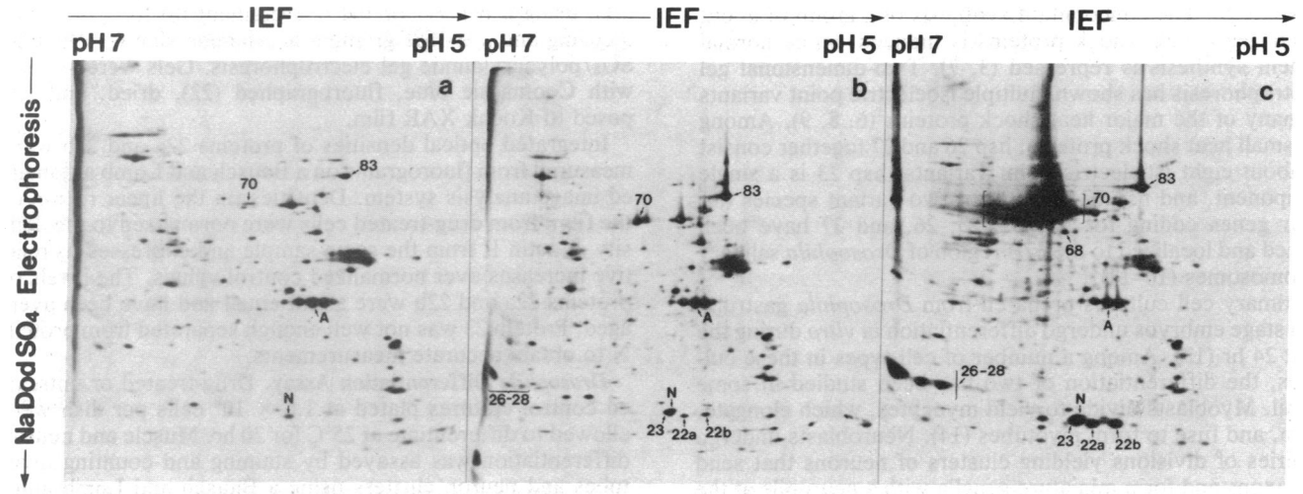

FIG. 2. Heat shock proteins in primary embryonic cells. *Drosophila* primary embryonic cells were allowed to differentiate at 25°C for 20 hr after plating. Cells were then labeled with [^{35}S]methionine for 1 hr at the indicated temperature, and proteins were separated by two-dimensional gel electrophoresis. The numbers indicate the major classes of heat shock proteins. A, actins; N, a non-heat shock protein that migrates near hsp 23 (8). Temperature and exposure times (cpm × days) were 25°C (223,000) (*a*); 30°C (307,000) (*b*); and 37°C (254,000) (*c*). IEF, isoelectric focusing.

mM), each of which inhibits muscle and neuron differentiation by >50%. Although the protein patterns are very similar in control and drug-treated samples, we observed a dramatic increase in the synthesis of three proteins, denoted 23, 22a, and 22b (23 and 22 kDa, respectively), in the drug-treated cells (Fig. 1). Primary embryonic cells treated with cortisone (0.01 mM), which does not inhibit differentiation in these cultures, synthesize low to moderate amounts of protein 23 and only trace amounts of proteins 22a and 22b, as do the untreated control cells.

To determine whether drugs that inhibit differentiation always induce the three proteins, we have tested a number of other drugs. To compare the effects of the drugs, integrated optical densities normalized to that of actin II were determined for proteins 22a and 22b. Table 1 summarizes the effects of each drug on embryonic differentiation and on the induction of proteins 22a and 22b.

Table 1. Effect of drugs on embryonic differentiation and the synthesis of proteins 22a and 22b

Treatment	Concentration, mM	*Drosophila* assay: Class	*Drosophila* assay: % of control: Neuron cluster	*Drosophila* assay: % of control: Myotube	Protein 22, relative increase over control
Control	—	—	100	100	1
5-Azacytidine	0.03	T	58	50	7
	0.3	T	28	32	86
Coumarin	1	T	19	7	64
Diphenylhydantoin	0.1	T	45	49	21
Pentobarbital	0.1	—	79	97	2
	1	T	78	48	25
Dexamethasone	0.1	T	54	38	10
Methyltestosterone	0.01	T	42	54	8
Diethylstilbestrol	0.01	T	82	48	7
β-Ecdysterone	0.01	T	32	51	7
Tolbutamide	1	T	45	30	6
Imipramine	0.01	—	73	89	2
	0.08	T	25	12	2
Amaranth	1	T	114	11	Trace
Amethopterin	1	T	53	45	0.4
Caffeine	1	NT	99	62	2
Antipyrine	1	NT	77	102	1
Dimethylsulfoxide	1	NT	129	95	0.6
Cortisone	0.01	NT	88	93	Trace
Saccharin	1	NT	68	112	0.8
Sulfanilamide	1	NT	94	95	0.2
Progesterone	0.01	NT	122	64	0.1
Ethanol	160 (0.95%)	NT	85	79	0.9

Drosophila primary embryonic cultures were allowed to differentiate at 25°C in the presence of the drug for 20 hr. Cultures were stained and numbers of differentiated myotubes and neuron clusters were counted. Results are expressed as a percentage of the number of myotubes and neuron clusters in control cultures prepared on the same day; most of these data were taken from Bournias-Vardiabasis *et al.* (18, 24) and are used here to illustrate the correlation between effects on embryonic differentiation and synthesis of protein 22. A drug was classified as a teratogen (T) in the *Drosophila* assay if, on three separate trials (four dishes per trial), there was at least 50% reduction in the number of myotubes or neuron clusters in the drug-treated cells compared to the control cells. NT, nonteratogen. Integrated optical densities for proteins 22a and 22b were determined from fluorograms using a Bausch and Lomb automated image analysis system. Values were normalized to the integrated optical density of actin II on the same film. The normalized values for proteins 22a and 22b were averaged and the results are expressed as relative increases over normalized untreated control values. Trace, trace amount, below sensitivity of image system.

Of the drugs that inhibit muscle or neuron differentiation in primary embryonic cell cultures, most induce the synthesis of proteins 23, 22a, and 22b. 5-Azacytidine (24), coumarin, diphenylhydantoin, and pentobarbital cause a 20- to 85-fold increase in the levels of proteins 22a and 22b. Dexamethasone, methyltestosterone, β-ecdysterone, diethylstilbestrol, and tolbutamide all give a 5- to 10-fold increase in the synthesis of these proteins.

Three drugs (amaranth, amethopterin, and imipramine) that inhibit embryonic differentiation do not appreciably induce any of the three proteins. Imipramine was too toxic to test at higher concentrations. Although these drugs certainly stress the cells, their mechanism of action may be substantially different from that of the other group of teratogens.

We have tested eight drugs that do not significantly inhibit muscle or neuron differentiation (including 0.95% ethanol, which is used as a solvent for some of the drugs) and have found no appreciable increase in the levels of proteins 23, 22a, and 22b compared to those of untreated controls.

Heat Shock Proteins in Embryonic Cells. What are the drug-induced proteins? Because their molecular weights are similar to those of the smallest heat shock proteins, we decided to examine the heat shock proteins produced by *Drosophila* primary embryonic cells after 20 hr of differentiation (Fig. 2). At 30°C and 37°C, the pattern of heat shock proteins, particularly the multiple components in the 70-kDa region and the 26- to 28-kDa region, corresponds closely to that observed in *Drosophila* salivary glands and continuous cell lines (8). At 25°C, hsp 83 is synthesized to an appreciable extent and several proteins in the 70-kDa region are synthesized at low levels.

In the primary embryonic cells, hsp 23, 22a, and 22b have electrophoretic mobilities on two-dimensional gels identical to those of the drug-induced proteins. The synthesis of hsp

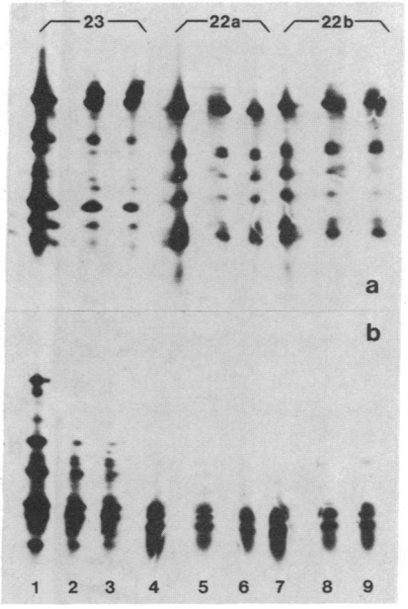

FIG. 3. Analysis of peptide fragments from heat shock and drug-induced proteins. [^{35}S]Methionine-labeled proteins from heat-shocked or drug-treated cells were separated by two-dimensional gel electrophoresis. The proteins were cut from the gels and digested with chymotrypsin (*a*) or *S. aureus* V8 protease (*b*). Peptides were separated on gels according to Cleveland *et al.* (23). Proteins 23, 22a, and 22b were obtained from cells heat shocked for 1 hr at 37°C (lanes 1, 4, and 7) or from cells treated for 20 hr with 0.1 mM diphenylhydantoin (lanes 2, 5, and 8) or 1 mM coumarin (lanes 3, 6, and 9).

22a and 22b is enhanced about 13-fold at 30°C and 75-fold at 37°C compared to that in 25°C control cells.

Comparison of the Drug-Induced Proteins with hsp 23, 22a, and 22b. To determine whether the drug-induced proteins are in fact hsp 23, 22a, and 22b, we compared the peptides from proteolytic digests of each of the three small heat shock proteins with each of the three drug-induced proteins from diphenylhydantoin- or coumarin-treated cultures. The peptide patterns for hsp 23, 22a, and 22b are essentially identical to the respective patterns from the drug-induced proteins (Fig. 3). Thus, we conclude that the drug-induced proteins are identical to or very closely related to hsp 23, 22a, and 22b.

Are hsp 26 and 27 Induced by Drugs? Genes for four small heat shock proteins (hsp 22, 23, 26, and 27) have been localized to the 67B region of the *Drosophila* chromosome (10–12). To determine whether all four of these proteins are induced by drugs in primary embryonic cells, we carried out two-dimensional gel electrophoresis of drug-treated samples by using a pH 6–8 gradient for isoelectric focusing, a procedure that clearly shows hsp 26 and 27.

In heat-shocked primary embryonic cells, there are multiple proteins in the 26- to 28-kDa region (Fig. 4 *c* and *d*). However, diphenylhydantoin-treated embryonic cells show no appreciable synthesis of hsp 26 or 27 on these gels (Fig. 4*b*). Cells treated with each of the other drugs listed in Table 1 were examined on pH 6–8 gels; none of the drugs induced hsp 26 or 27 (data not shown).

Effect of β-Ecdysterone on Primary Embryonic Cell Cultures. β-Ecdysterone, the *Drosophila* molting hormone, induces the synthesis of all four of the small heat shock proteins or their transcripts in certain *Drosophila* cell lines (25) and in imaginal discs (26). In primary embryonic cells, we found that it inhibits both muscle and neuron differentiation at concentrations between 0.01 mM and 0.1 μM; it is cytotoxic at 0.1 mM. β-Ecdysterone also induces hsp 23, 22a, and 22b but not hsp 26 or 27 (Table 1). The reason for the difference in response to β-ecdysterone by the primary embryonic cells compared to imaginal discs or cell lines is not clear. However, the primary embryonic cells, which would not be exposed to high levels of β-ecdysterone *in vivo*, respond to the hormone as they do to other drugs that stress the cells.

Dose–Response Studies. To investigate further the correlation between drug effects on embryonic cell differentiation and the induction of hsp 22a and 22b, we carried out dose–response studies with diphenylhydantoin and coumarin. Fig. 5 shows that increasing concentrations of either drug decrease the amount of muscle and neuron differentiation and increase the synthesis of hsp 22a and 22b. Cells are sensitive to diphenylhydantoin at concentrations about 1/5th to 1/2 of the levels of coumarin required for similar effects. Pentobarbital and 5-azacytidine also show a concentration-dependent response for both differentiation and induction of hsp 22a and 22b (Table 1).

DISCUSSION

Nine of 12 drugs that inhibit muscle or neuron differentiation in embryonic *Drosophila* cells also induce three heat shock proteins, hsp 23, 22a, and 22b. Whether these proteins play any direct role in the process of teratogenesis is unknown. It is perhaps more likely that their presence may be an indication of cellular stresses likely to give rise to teratogenesis. We have previously shown that the teratogenic effects of diphenylhydantoin or coumarin in primary embryonic cells are partially alleviated by mild heat pretreatment, which induces the full set of heat shock proteins (19).

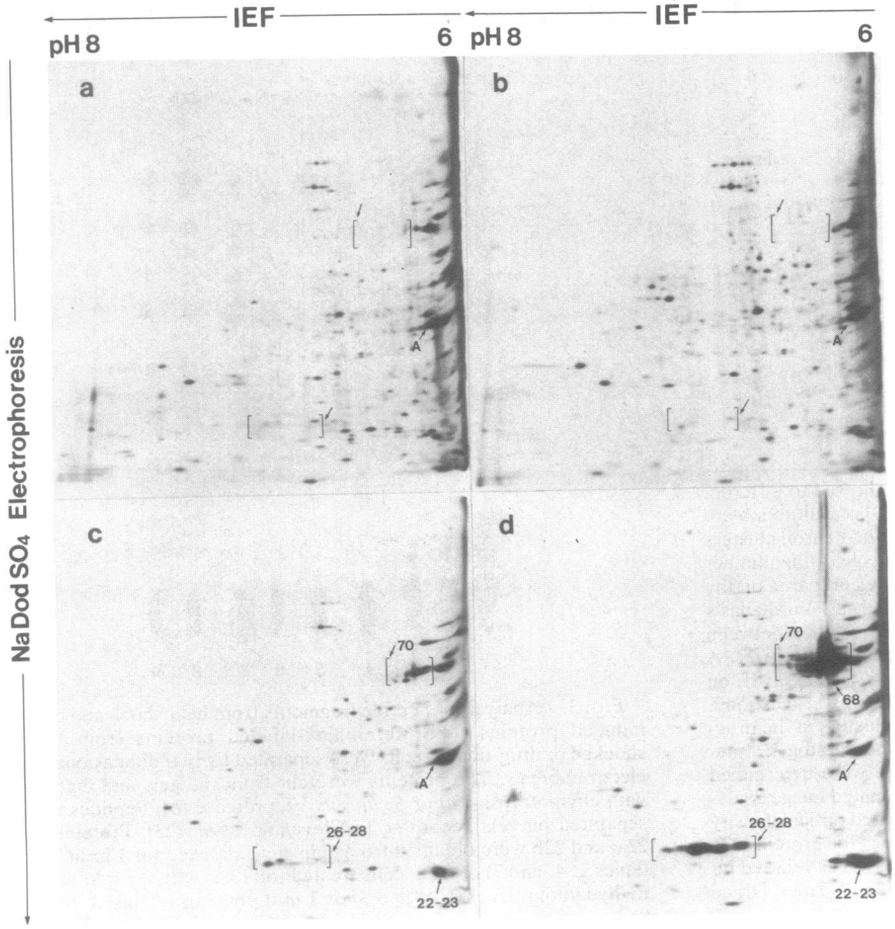

FIG. 4. Induction of hsp 26 and 27. *Drosophila* primary embryonic cells were treated with drugs or heat, labeled with [^{35}S]methionine, and solubilized as described in the legends to Figs. 1 and 2. Proteins were separated in the first dimension on a pH 6–8 gradient. The second dimension, $NaDodSO_4$/polyacrylamide gel electrophoresis, was the same as in Figs. 1 and 2. (*a*) Control (686,000 cpm × days); (*b*) diphenylhydantoin, 0.1 mM (625,000 cpm × days); (*c*) heat shock, 30°C, 1 hr (303,000 cpm × days); (*d*) heat shock, 37°C, 1 hr (254,000 cpm × days). IEF, isoelectric focusing.

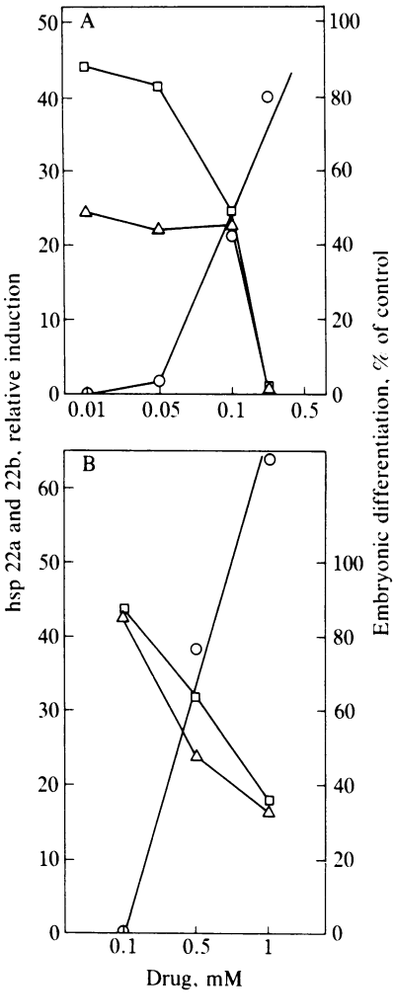

FIG. 5. Concentration-dependent effects of diphenylhydantoin and coumarin. Muscle and neuron differentiation of *Drosophila* primary embryonic cells in the presence of a given drug was determined as described in Table 1. Results are expressed as a percentage of the numbers of myotubes and neuron clusters in untreated cells. The relative induction of hsp 22a and 22b was determined by measuring integrated optical densities on fluorograms, as described in Table 1. Results are expressed as a relative increase in the normalized integrated optical densities of hsp 22 in drug-treated cells compared to those of untreated cells. (*A*) Diphenylhydantoin; (*B*) coumarin. □, Myotube differentiation; △, neuron differentiation; ○, relative induction of hsp 22a and 22b.

It has recently been shown that some of the small heat shock proteins may play a role in normal development as well as in the heat shock response. In late third instar larvae and prepupae, hsp 22, 23, 26, and 27 or their transcripts have been detected (26–28). At a different stage of normal development, mRNAs for hsp 26 and 28/27 accumulate in adult ovaries and are abundant in early embryos until about blastoderm stage (29).

Drug induction provides one of the few cases in which only a subset, rather than the full set, of heat shock proteins is synthesized. Our results show that the synthesis of the four small heat shock proteins can be regulated separately, even though the genes for all are closely linked. Drug induction may be used to study the stress reaction in cells that leads both to the production of heat shock proteins and teratogenic defects. In addition, studies on the function and control of hsp 22 and 23 can be carried out in the absence of the other heat shock proteins.

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