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Genetics

Synthesis of low molecular weight heat shock peptides stimulated by ecdysterone in a cultured *Drosophila* cell line

(two-dimensional gel electrophoresis/one-dimensional peptide map/steroid hormone/gene expression)

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ABSTRACT **Treatment of Schneider's line 3 *Drosophila* cells with the steroid hormone ecdysterone rapidly stimulated the synthesis and accumulation of the polypeptide previously designated p7 [Berger, E. M., Ireland, R. C. & Wyss, C. (1980) *Somatic Cell Genet.* 6, 119–129]. In this report, p7 is identified as the 23,000-dalton heat shock polypeptide (hsp23). In addition to hsp23, the synthesis of the low molecular weight heat shock polypeptides hsp22, hsp26, and hsp27 was also stimulated by ecdysterone, although to different extents. Hybridization of a nick-translated genomic clone containing the hsp23 gene to a total RNA blot showed that ecdysterone stimulation of hsp23 synthesis was the result of an increase in the hsp23 RNA content of S3 cells. We detected no effect of the hormone on the synthesis of heat shock polypeptides hsp68, hsp70, and hsp83.**

Cultured embryonic *Drosophila* cell lines have been used extensively for studies of gene regulation mediated by the steroid hormone ecdysterone (1–8) and by heat shock (9–12).

The study of ecdysterone-regulated gene expression has largely involved the analysis of hormone-induced changes in morphology and motility (3, 13, 14) and changes in the spectrum of proteins and enzymes synthesized after ecdysterone treatment (3, 6, 7, 15, 16). Recently, Savakis *et al.* (17) isolated cDNA clones containing the coding sequences for three ecdysterone polypeptides in the Kc cell line.

The heat shock phenomenon has been studied in both *Drosophila* larvae and tissue culture cells (9–12) (for review, see ref. 18). Genomic clones for the major heat shock genes have been isolated and the molecular structures and arrangements of these genes have been examined in detail (19–23).

The present report concerns the stimulation of the synthesis of a polypeptide in Schneider's line 3 (S3) *Drosophila* cells by ecdysterone. We have previously designated this polypeptide p7 (7); in this report, we identify p7 as the 23,000-dalton heat shock polypeptide (hsp23). In addition, we show that ecdysterone also stimulates the synthesis of three other low molecular weight heat shock polypeptides—hsp22, hsp26, and hsp27—without influencing the expression of the major heat shock polypeptides hsp68 and hsp70.

MATERIALS AND METHODS

Cell Culture and Labeling. Schneider's line 3 (S3) cells were grown in Shields and Sang medium (24) as described (7). Ecdysterone (20-hydroxyecdysone, Calbiochem) was added from an aqueous stock solution (1 mg/ml) to 21.5 μM unless otherwise indicated, and cells were incubated at 25°C for 22 hr. Protein labeling was as described (7), except that the labeling time was reduced to 2 hr. For heat shock experiments, cells were incubated 30 min at 37°C before the addition of [^{35}S]methionine.

Electrophoresis and Autoradiography. Protein extraction and two-dimensional [isoelectric focusing (IEF)/NaDodSO$_4$] polyacrylamide gel electrophoresis were carried out according to the procedure of O'Farrell (25). Expansion of the isoelectric focusing dimension was accomplished by reversing the Ampholytes (LKB) pH 5–7/pH 3.5–10 ratio of 4:1. Second dimension gels (17.5%) were stained with Coomassie blue, dried, and exposed to Kodak NS-5T x-ray film. Quantification of the two-dimensional gels was as described (7).

One-Dimensional Peptide Mapping. Polypeptides of interest were excised from lightly stained two-dimensional gels by using a no. 2 cork borer and stored in buffer A (0.125 M Tris, pH 6.8/0.1% NaDodSO$_4$/1 mM EDTA) at −20°C. Digestion with *Staphylococcus aureus* V8 protease (Miles) was carried out according to Cleveland *et al.* (26), and peptide fragments were visualized by fluorography.

Quantification of hsp23 RNA. Total cellular RNA was prepared from ecdysterone-treated S3 cells by the method of Scott *et al.* (27) and dot blotted onto nitrocellulose. A *Drosophila* genomic DNA fragment containing the hsp23 gene cloned in pBR322 (the gift of E. A. Craig) was labeled with [^{32}P]dCTP by nick translation and hybridized to the RNA blot (28). After removal of nonspecifically bound probe, the blot was exposed to NS-5T film at −80°C.

RESULTS

Induction of p7 by Ecdysterone. S3 *Drosophila* cells respond to 10 μM ecdysterone by increasing the synthesis of the low molecular weight polypeptide (Fig. 1) previously designated p7 (7). To quantitate this increase in p7 synthesis and accumulation, we compared the [^{35}S]methionine cpm in p7 with the sum of the [^{35}S]methionine cpm in a set of reference polypeptides as described (7). The reference polypeptides were chosen on the basis of their apparent invariance, as judged qualitatively from autoradiograms. Results of three independent experiments showed that p7 synthesis was stimulated an average of 14-fold above control cell levels by ecdysterone treatment (Table 1).

To determine the optimum ecdysterone concentration for p7 induction, S3 cells were treated with ecdysterone at concentrations ranging from 10 μM to 1 nM for 24 hr. After cell labeling and two-dimensional gel electrophoresis, the extent of p7 induction was determined and compared with that of control cells. Maximum stimulation of p7 synthesis occurred at 0.1–10 μM ecdysterone. At concentrations lower than this, the response rapidly diminished, and it was absent below 1 nM. Two other steroids, testosterone and estradiol, were ineffective in stimulating p7 synthesis at 21.5 μM.

Kinetics of the Stimulation of p7 Synthesis. We next determined the kinetics of p7 induction, because a rapid response

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Abbreviations: hsp, heat shock polypeptide; IEF, isoelectric focusing.

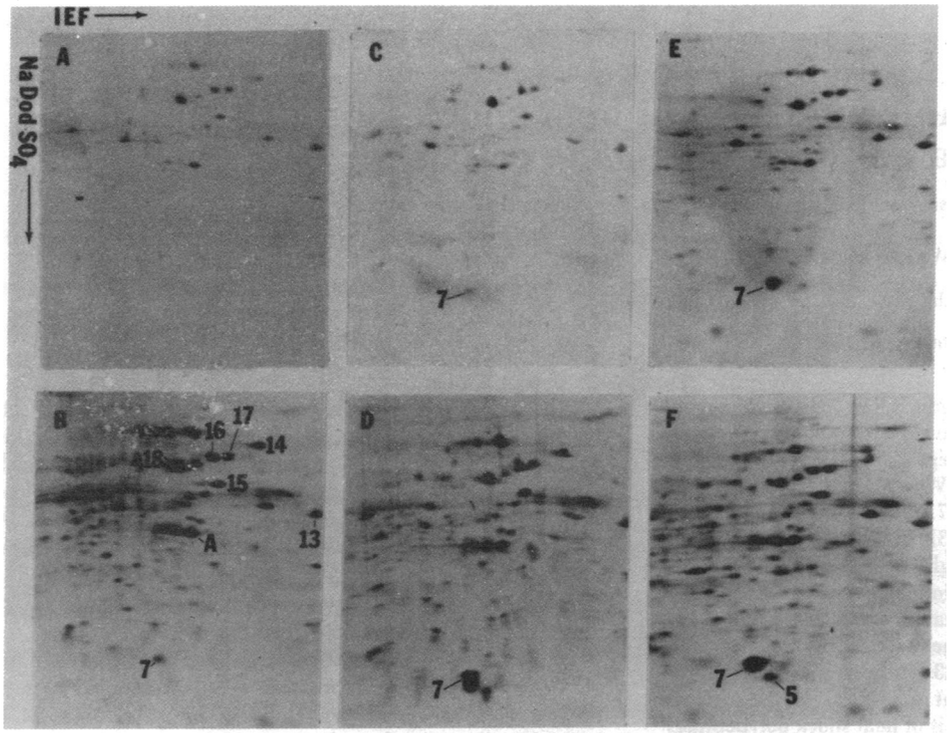

FIG. 1. Two-dimensional gel electrophoretic pattern of [^{35}S]methionine-labeled S3 cells showing the stimulatory effect of ecdysterone on p7 synthesis. Cells were treated for 0 (*A* and *B*), 24 (*C* and *D*), or 72 (*E* and *F*) hr with 21.5 μM ecdysterone. (A, *C*, and *E*) Coomassie blue staining patterns. (*B*, *D*, and *F*) The corresponding autoradiograms.

is generally indicative of a primary hormone effect. When S3 cells were treated with hormone for up to 4 hr (including the labeling time), no increase in p7 synthesis could be detected until after the first hr (Fig. 2).

Identity of Low Molecular Weight Ecdysterone-Induced Polypeptides. The position and orientation of p7 and the nearby polypeptide designated p5, whose synthesis was also stimulated somewhat by ecdysterone (see Fig. 1*F*), resembled the position and orientation of the low molecular weight heat shock polypeptides hsp23 and hsp22, respectively (29). To confirm our suspicions, we compared the two-dimensional gel patterns of polypeptides labeled at 37°C and polypeptides labeled in the absence and presence of ecdysterone at 25°C. An extended pH gradient was established in the first-dimension gels so that the survey would also include the low molecular weight heat shock polypeptides hsp26 and hsp27. The results (Fig. 3) demonstrate two things. First, the positions of the four polypeptides induced by heat shock correspond well with the positions of the four polypeptides whose synthesis was stimulated by ecdysterone. In fact, when cell lysates from hormone-stimulated and heat shock S3 cells were mixed, the ecdysterone-induced polypeptides p5 and p7 comigrated with hsp22 and hsp23 respectively (data not shown). Second, although it is clear from the gels in Fig. 3 and subsequent quantitative data (Table 2) that ecdysterone stimulated the synthesis of four small heat shock polypeptides, the major heat shock polypeptides—hsp68 and hsp70—were never induced by the hormone.

Comparison of p7 and hsp23 by One-Dimensional Peptide Mapping. We further investigated the relationship between p7 and hsp23 by comparing the one-dimensional peptide maps generated by digestion of the polypeptides with *Staphylococcus* V8 protease (26). We found that the patterns of peptide fragments generated by digestion of p7 and hsp23 were identical (Fig. 4). We also found that cell lysates from which p7 was obtained for digestion contained no high molecular weight heat shock polypeptides, indicating that the induction of p7 synthesis by ecdysterone in these cells represents a true hormone response rather than a transient heat shock.

Table 1. Stimulation of p7 synthesis by ecdysterone

Peptide	Relative rate of synthesis		
	Control	Ecdysterone	E/C
13	7.0	4.3	0.614
14	14.6	12.2	0.836
15	10.4	8.1	0.779
16	13.2	9.5	0.720
17	11.5	4.4	0.383
18	21.0	20.6	0.981
Actin	22.4	41.0	1.830
7	2.8	39.8	14.214

Results are expressed as (peptide cpm/reference cpm) × 100. C, control; E, ecdysterone.

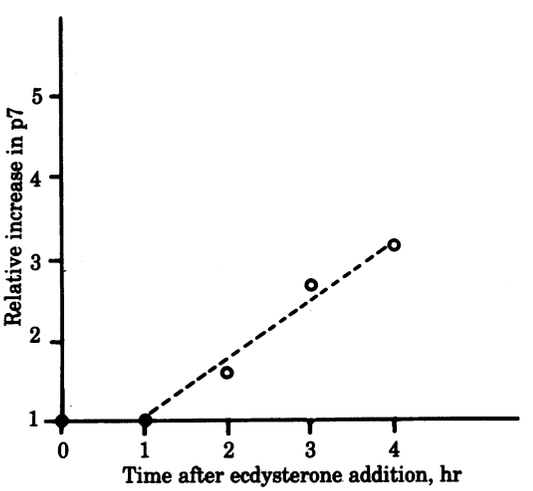

FIG. 2. Kinetics of stimulation of p7 synthesis by ecdysterone. S3 cells were treated with 21.5 μM ecdysterone for various lengths of time, labeled with [^{35}S]methionine, and analyzed by two-dimensional gel electrophoresis.

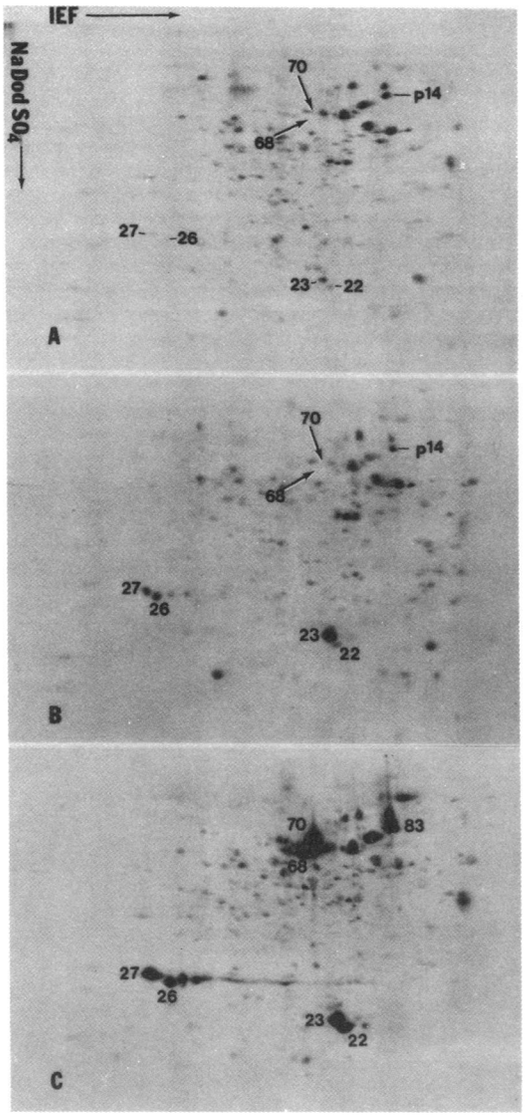

FIG. 3. Comparison of positional orientations of four low molecular weight polypeptides whose synthesis is stimulated by both ecdysterone and heat shock. Two-dimensional gel patterns of S3 cells labeled in the absence (*A*) and presence (*B*) of ecdysterone and during heat shock (*C*). An expanded pH gradient was used in the IEF dimension to resolve the 26,000- and 27,000-dalton heat shock polypeptides.

Absence of p7 Induction in Ecdysterone-Resistant Cell Lines. After we had established that p7 was hsp23 and p5 was hsp22, we questioned whether their induction by ecdysterone represented a *bona fide* hormone response, or reflected a reaction to stress. We could rule out a general stress response, like heat shock, for several reasons. First, there was never any induction of p7 in sham-treated control, in cells treated with very low concentrations of ecdysterone, or in cells treated with other steroids such as testosterone and estradiol. Because the procedures of cell harvesting, incubation, and radiolabeling were all the same, the single variable was ecdysterone concentration. Moreover, the synthesis of hsp68 and hsp70 was never stimulated by hormone treatment.

The remaining possibility is that ecdysterone, at concentrations >1 nM, represents a specific, but mild, stress that stimulates the synthesis of p7. The experiments described below show, in fact, that the reverse is true.

We examined p7 inducibility in a fusion hybrid cell line constructed with S3 and the hormone-responsive cell line MDR.

Table 2. Stimulation of synthesis of low molecular weight heat shock polypeptides by ecdysterone

Peptide	Relative rate of synthesis		
	Control	Ecdysterone	E/C
hsp22	0.6	1.0	1.667
hsp23	1.9	24.2	12.737
hsp26	0.8	8.1	10.125
hsp27	1.2	3.7	3.080

Results are expressed as (peptide cpm/reference cpm) × 100. C, control; E, ecdysterone.

The resultant hybrid line, designated SM3 (7, 30) showed a substantial level of p7 induction, which was intermediate between the parental lines (Table 3). SM3 is fully responsive to ecdysterone by a variety of criteria (7, 30).

A series of fusion hybrid cell lines was constructed between S3 and MDER, an ecdysterone-resistant derivative of MDR (31), and these hybrids fell into two phenotypic classes. In lines F1 and F6, ecdysterone resistance was dominant and p7 synthesis was not stimulated (Table 3 and Fig. 5). Line F7 proved ecdysterone sensitive and p7 synthesis was stimulated by hormone treatment (Table 3).

In summary, p7 inducibility appears to be invariably associated with ecdysterone responsiveness. In control experiments, we verified that each of the parental and hybrid cell lines retained the normal heat shock response and, in addition, we discovered an electrophoretic mobility variant of hsp22 (Fig. 5).

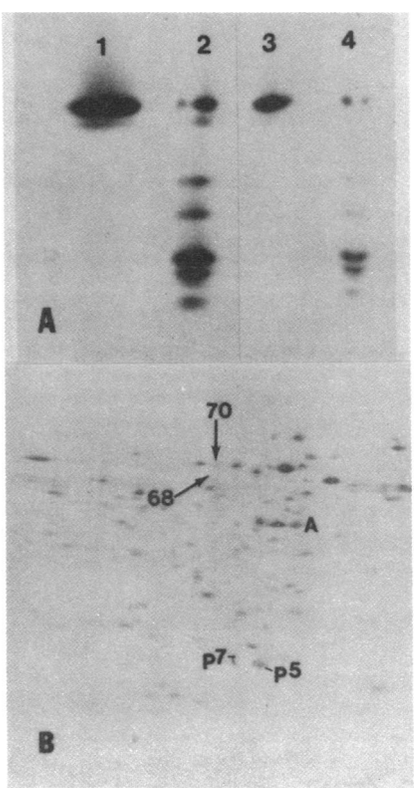

FIG. 4. Comparison of p7 and hsp23 by one-dimensional peptide mapping. (*A*) hsp23 (lanes 1 and 2) and p7 (lanes 3 and 4) were obtained from second-dimension gels of heat shocked and ecdysterone-treated S3 cells, respectively, and digested (lanes 2 and 4) with 1 μg of *Staphylococcus* V8 protease as described (26). Peptide fragments were visualized by fluorography. (*B*). Autoradiogram of a second-dimension gel from which p7 was obtained for digestion experiments showing the absence of hsp68 and hsp70 from the cell lysate used in these experiments.

Table 3. Relative rates of p5 and p7 synthesis in the presence or absence of ecdysterone for cell lines and their hybrids

	p7		p5a		p5b	
Cell line	Control	Ecdysterone	Control	Ecdysterone	Control	Ecdysterone
S3	12.5 ± 0.3	129.4 ± 3.0	3.5 ± 0.5	13.8 ± 0.2		
MDR	7.0 ± 0.6	10.9 ± 0.4			1.8 ± 0.7	4.2 ± 0.2
MDER	6.8 ± 0.5	5.2 ± 0.4			1.4 ± 0.3	1.0 ± 0.4
SM3	5.9 ± 0.2	25.8 ± 0.8	2.5 ± 0.1	7.9 ± 0.2	1.7 ± 0.1	5.5 ± 0.2
F1	4.7 ± 0.9	5.2 ± 0.2	1.7 ± 0.4	1.9 ± 0.1	1.5 ± 0.7	1.3 ± 1.1
F7	5.0 ± 0.8	15.8 ± 0.3	2.5 ± 0.2	4.5 ± 0.3	1.3 ± 0.7	3.3 ± 0.6

Results are mean ± SEM. Details of the calculations in this table are given in ref. 7. Note that p6 in ref. 7 has been renumbered p5b and p5 is now p5a.

The hybrid lines all showed a codominant heat shock phenotype with respect to hsp22 and, in hormone-responsive hybrid cell lines, both forms of hsp22 were induced by ecdysterone (Fig. 5 and Table 3).

Quantification of hsp23 RNA in Ecdysterone Stimulated Cells. It was of interest to us to distinguish whether ecdysterone stimulated the synthesis of hsp23 by increasing the amount of hsp23 RNA in the cells or by increasing the rate at which preexisting hsp23 RNA was translated. We addressed this question by determining the amount of hsp23 RNA in S3 cells as a function of time after beginning ecdysterone treatment, assuming that transcriptional control would result in an increase in the cellular content of hsp23 RNA while translational control would not. Total S3 RNA from control cells and from cells treated for various lengths of time with 10 μM ecdysterone was bound to nitrocellulose (28) and probed with a nick-translated recombinant plasmid containing the hsp23 coding sequence. The results showed that the relative amount of RNA complementary to the hsp23 sequence increased as the length of the ecdysterone treatment increased (Fig. 6).

DISCUSSION

Treatment of Schneider's line 3 (S3) *Drosophila* cells with ecdysterone stimulated the synthesis of the polypeptide previously designated p7 (7). In this report, we have presented evidence identifying p7 as the 23,000- dalton heat shock polypeptide, hsp23. Furthermore, we have shown that the synthesis of three

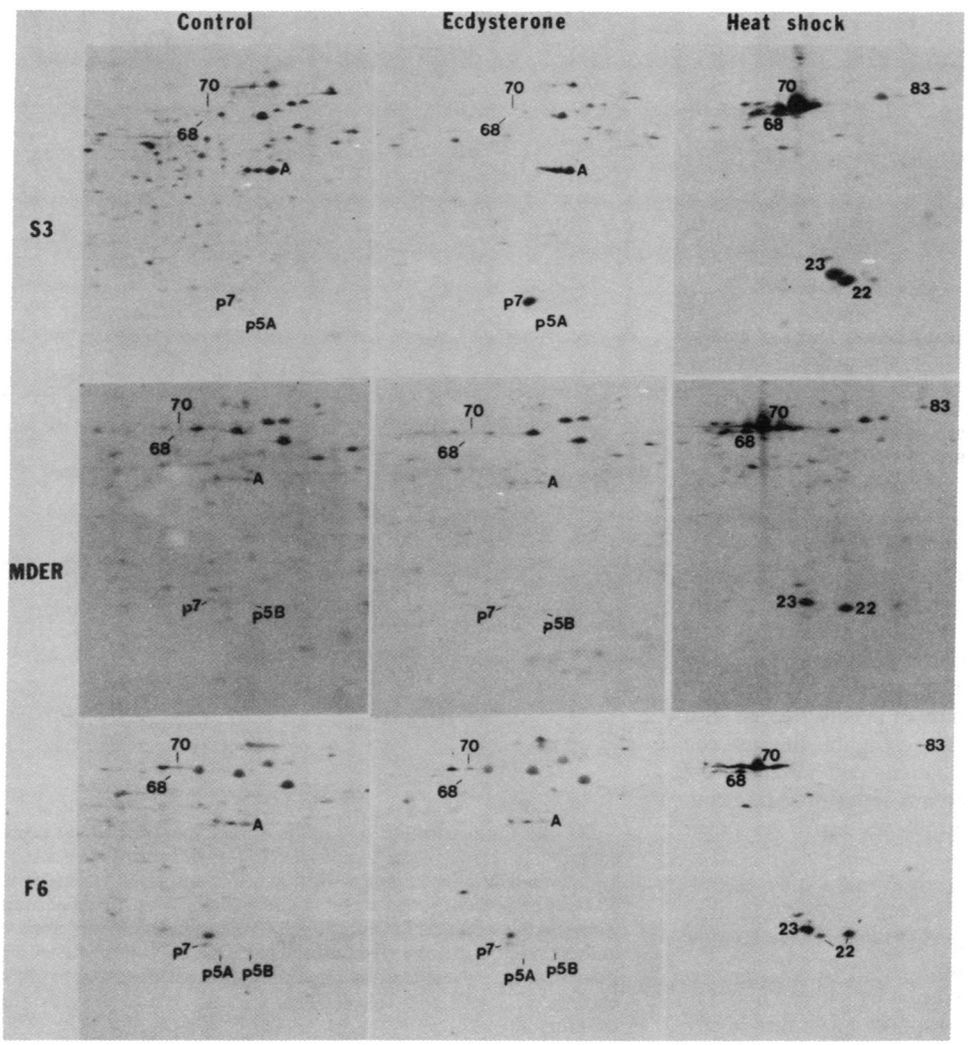

FIG. 5. Two-dimensional electrophoretic patterns of polypeptides synthesized in cells resistant to the growth-inhibiting effect of ecdysterone. Cell lines used were S3 (ecdysterone sensitive), MDER (ecdysterone resistant), and F6 (a fusion hybrid of S3 and MDER that is ecdysterone resistant). This figure also illustrates the electrophoretic mobility polymorphisms detected in hsp22 (compare S3 and MDER).

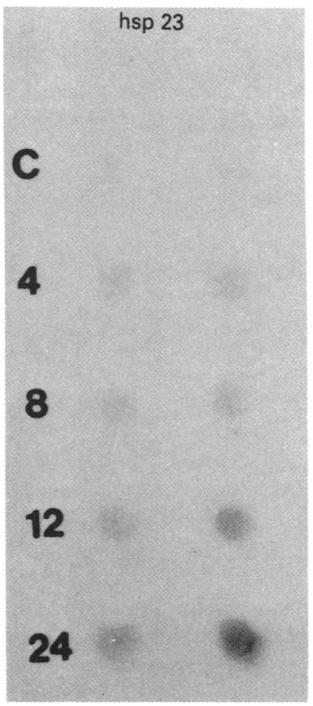

FIG. 6. Quantification of hsp23 RNA in ecdysterone-treated S3 cells by hybridization of a genomic clone of the hsp23 gene to total RNA dot blotted onto nitrocellulose (28). RNA was isolated from S3 cells treated for various lengths of time with ecdysterone and blotted in either 1 μg (*Left*) or 5 μg (*Right*) amounts. Results are relative amounts.

other low molecular weight heat shock polypeptides, hsp22, hsp26, and hsp27, is also stimulated by ecdysterone in this cell line.

Stimulation of p7 synthesis in S3 cells by ecdysterone could be detected after 1 hr in the presence of hormone, suggesting that the response could be the result of a primary hormone effect. The optimal hormone concentration for eliciting the response was 0.1–10 μM, which is within the normal physiological range (8, 32).

We have identified p7 as the 23,000-dalton heat shock polypeptide (hsp23) on the basis of comigration of p7 and hsp23 on two-dimensional gels and by one-dimensional peptide mapping. In an earlier report, this identity was missed because, at the temperature at which the heat shock experiments were performed (38°C), induction of the low molecular weight heat shock polypeptides fails to occur (33).

Ecdysterone treatment of S3 cells also stimulated the synthesis of three other low molecular weight heat shock polypeptides, hsp22, hsp26, and hsp27, although to different extents (Table 2). Thus, although the genes coding for these four polypeptides are closely linked (22, 23) and despite the fact that they appear to be regulated coordinately by ecdysterone and heat shock, their protein products are not equally represented in ecdysterone-treated S3 cells.

It is our feeling that the induction described is not a result of stress generated by ecdysterone or by the labeling conditions (34). The stimulatory effect shows a dose–response profile characteristic of an authentic hormone response, and hsp23 and hsp22 induction fails to occur in cells that have acquired resistance to the growth-inhibiting effects of ecdysterone by direct selection or fusion with hormone-resistant cell lines (Fig. 6).

Analysis of the effect of ecdysterone on hsp23 RNA accumulation by hybridization of a nick-translated genomic hsp23 DNA clone to total RNA bound to nitrocellulose indicated that treatment of S3 cells with ecdysterone resulted in an accumulation of hsp23 RNA in the cells. This is suggestive of a regulatory effect of the hormone at the level of RNA transcription, although we have not ruled out an effect of the hormone on hsp23 RNA stability.

It has recently been brought to our attention that polyadenylated RNA from late third instar/early pupal stage *Drosophila* larvae contain substantial amounts of sequences complementary to the hsp22 and hsp26 genes (35). Because this stage of *Drosophila* development is characterized by high ecdysterone titers (36), this finding lends support to our hypothesis.

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