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Developmental Changes of HSP23 Gene Expression and 20-Hydroxyecdysone Synthesis in *Drosophila melanogaster*

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The heat shock protein (HSP) genes are expressed at various stages of the *Drosophila* life cycle even under non-heat-shock conditions. In the present study, developmental changes of HSP23 gene expression and the role of 20-hydroxyecdysone (20HE) on the HSP23 gene expression were investigated in *Drosophila melanogaster*. The Northern blot and Western blot analyses showed that HSP23 gene expression occurred at the early third instar larval stage, reached the highest with a sharp peak in the white prepupa, and then decreased throughout the pupal period. When the HSP23 gene expression was compared with the secretion of 20HE, there is a similarity between 20HE synthesis and HSP23 gene expression during the third instar larval-prepupal period. It appears that 20HE regulates expression of HSP23 gene at larva-pupa molting period, and that, 20HE is also involved in the control the metamorphosis in some part through the HSP23.

KEY WORDS: *Drosophila melanogaster*, HSP23, 20-hydroxyecdysone, Developmental regulation

The first evidence that various kinds of stress lead to the activation of Heat shock protein (HSP) genes came from the observation of Ritossa (1962) that some new puffs appeared on the *Drosophila* giant salivary gland chromosomes after heat shock at 37°C or following treatment of dinitrophenol or sodium salicylate. Some HSPs are synthesized developmental stage-specifically in the *Drosophila* life cycle even under non-heat-shock conditions (Zimmerman *et al.*, 1983; Chung *et al.*, 1989). Most HSPs are expressed at one time or another during normal development in *Drosophila*. The presence of HSPs under normal conditions suggests that they have a function under the physiological conditions.

Drosophila cells of established lines undergo the hormone-induced changes both at the morphological level and at the level of gene expression. The low molecular weight HSPs are induced by the molting hormone, 20-hydroxyecdysone (20HE), in cultured cells. Ireland and Berger (1982) reported that the synthesis of HSP23 and other low molecular weight HSPs such as HSP22, HSP26 and HSP27 but not the HSP83 and HSP70 was stimulated by 20HE, although to different extents. Northern blot hybridization also revealed that stimulation of HSP23 synthesis by 20HE was the result of an increase in the HSP23 RNA content in Schneider's line 3 (S3) *Drosophila* cells (Ireland *et*

al., 1982).

The presence of small HSP mRNAs at pupariation and pupation, and increase of these mRNAs in cultured cells and isolated imaginal discs treated with 20HE (Ireland and Berger, 1982; Ireland *et al.*, 1982; Vitek and Berger, 1984) suggest a possibility that developmental regulation of the genes encoding small HSPs is induced by 20HE during normal development.

In the present study, we investigated changes in HSP23 gene expression and 20HE synthesis during development of *Drosophila melanogaster*. Also, the functional relationship between HSP23 and 20HE was investigated.

Materials and Methods

Culture of *Drosophila*

The Or-R wild type of *D. melanogaster* was reared in half-pint glass bottles containing the medium (cornmeal, sugar, agar, yeast and propionic acid) at 24°C under a photoperiodic regime (12L:12D). Synchronization of developmental stages was achieved according to the method of Ashburner and Tompson (1978).

Purification, antiserum preparation and Western blotting of HSP23

The late third instar larvae were heat-treated at 37°C for 1 hr, homogenized and then centrifuged at 10,000 ×g for 10 min. The supernatant was removed and fractionated by ammonium sulfate precipitation. The fraction containing HSP23 was electrophoresed on SDS-polyacrylamide gel, and then the HSP23 band was sliced. The gel slice was homogenized with an equal volume of phosphate buffered saline (PBS), and then injected subcutaneously into a rabbit. After three booster injections of partially purified HSP23 at 2-week intervals, the blood was collected by heart puncture and tested for specific antibody with double diffusion. The blood was stored as a solution at -70°C in the presence of 0.02% sodium azide. The HSP23 was quantified by Western blot analysis using 1:250 dilution of anti-HSP23 antiserum followed by 1:500 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma).

Northern blot analysis of HSP23 mRNA

The HSP23 gene probe, isolated from plasmid aDm202.7 (gifted from Dr. J. Lis at Cornell University, Costlow and Lis, 1984: Fig. 2A), was labeled with α-^{32}P-dCTP (spec. act. 3,000 Ci/mmol, Amersham) using random priming method (Boehringer Mannheim). Total RNA was isolated from 10-20 individuals at particular developmental stages as described by Chomczynski and Sacchi (1987) and the concentrations were determined spectrophotometrically. The RNA was separated on 1% formaldehyde agarose gel and transferred onto nitrocellulose filters. The filters were prehybridized with prehybridization buffer (5X SSC, 50% formamide, 5X Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA) at 42°C for 4 hrs, and then hybridized with hybridization buffer (5X SSC, 50% formamide, 1X Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA) containing α-^{32}P-labeled gene probe at 42° C for 24 hrs. The filters were exposured to X-ray film at -70°C for 1-3 days.

Preparation of anti-20HE antiserum and radioimmunoassay (RIA)

The immunogen for 20HE was synthesized by the method of Maroy *et al.* (1977) and Reum *et al.* (1981). 20HE was converted into oxime form from reaction with carboxymethoxilamine hemihydrochloride (20HE-6-carboximethoxime), and then covalently coupled to bovine serum albumin (20HE-6-carboximethoxime-BSA). Anti-20HE antiserum was prepared by the similar method used in the production of anti-HSP23 antiserum.

Ecdysteroids was quantified by RIA with charcoal absorption separation method using antiserum prepared in the present study or DBL-2 (gifted from Dr. J. Koolman at Marburg University, Reum *et al.*, 1981) and [23,24-^{3}H] ecdysone (spec. act. 89 Ci/mmol, New England Nuclear). A standard curve was generated using different amounts of ecdysone (working range: 15-500 pg).

Results and Discussion

Preparation of HSP23 and 20HE antisera

HSP23 was partially purified by the simple steps as shown in Fig. 1A. The purified HSP23 was subcutaneously injected into a rabbit. The production of anti-HSP23 antibody was tested by a double diffusion experiment (data not shown). The HSP23 was strongly detected not only in the purified HSP23 but also in the salivary glands, when the specificity was tested by Western blot analysis using an 1:250 dilution of antiserum (Fig. 1B). However, some other small HSPs such as HSP22, 26 and 27, were weakly cross-reacted. It may be due to the high homologies between HSP23 and other small HSPs (Ingolia and Craig, 1982).

The immunogenic substance for 20HE (20HE-6-carboxymethoxime-BSA) was prepared using carboxymethoxilamine hemihydrochloride and BSA as spacer and carrier protein, respectively. The coupled number of 20HE-oxime per BSA was measured to about five. The prepared immunogen was injected into rabbit and then the antiserum was obtained by the same method used in the preparation of anti-HSP23 antiserum.

The titers and the steroid specificities of antiserum were analyzed by RIA using [23,24-^{3}H] ecdysone as tracer. From the standard curves, the concentrations were taken at which 50% of the radiolabeled ecdysone bound to antibodies was displaced. A comparison of these concentrations between ecdysteroids tested and ecdysone gave the cross reaction factors (C_f). When the specificity of this antiserum was measured against several ecdysteroids, the cross reaction factor (C_f) of 20HE and 20HE-22-acetate with ecdysone were 0.7 and 57.5, respectively. This antiserum was immunoreactive for ecdysone and 20HE with a similar sensitivity, but a slightly more sensitive to 20HE. Thus, it was regarded that the steroid amount measured by RIA using the antiserum prepared may include a sum of ecdysone and 20HE (some other ecdysteroid species could be

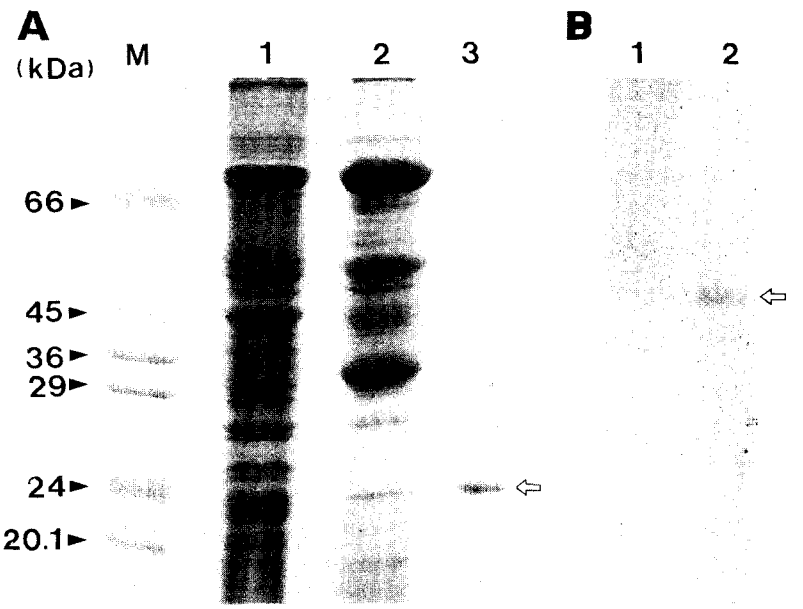


Fig. 1. Purification of HSP23 and detection of HSP23 by Western blot analysis. HSP23 was partially purified from the late third instar larvae and used as immunogen. The arrows indicate HSP23. (A) SDS polyacrylamide gel electrophoresis of each purifing step of larval homogenate. M, standard marker; 1, larval extract; 2, 70-100% saturated ammonium sulfate fraction; 3, homogenate of sliced HSP23 band. (B) Detection of HSP23 by Western blot analysis. 1, salivary gland proteins; 2, purified HSP23.

also measured).

Developmental changes of HSP23 gene expression

The relative amount of HSP23 transcripts during development was determined by Northern blot analysis. As shown in Fig. 2, the HSP23 mRNA was begun to increase in the early third instar larva. HSP23 mRNA level was increased from this stage and reached the highest with a sharp peak at the white prepupal stage. After then, it was decreased throughout the pupal period with a minimum detection in adult. The sharp increase followed by decrease of HSP23 gene expression was noticeable during the short-termed (2-3 hrs) white prepupal period.

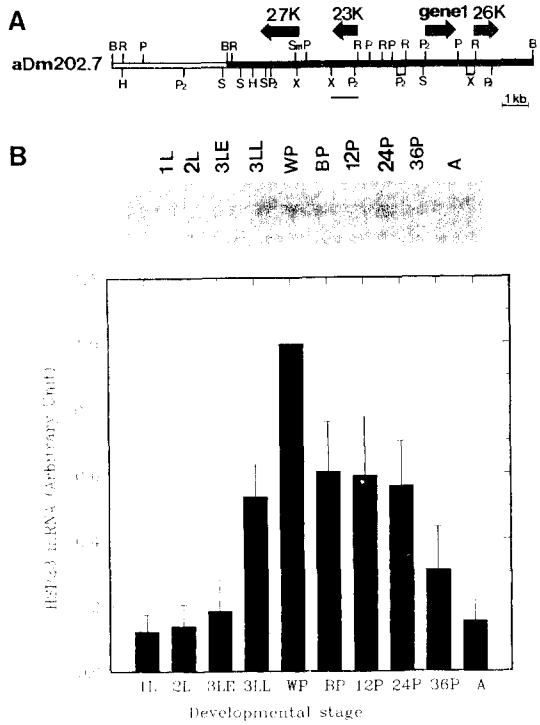


Fig. 2. Developmental analysis of HSP23 gene expression by Northern blotting. (A) Restriction map of plasmid aDm202.7. An 1 kb DNA fragment containing HSP23 gene (underlined region) was used as probe. B, BamH I; H, Hind III; P, Pst I; P_2, Pvu II; R, EcoR I; S, Sal I; Sm, Sma I; X, Xba I. (B) Northern blot analysis of HSP23 mRNA. Relative abundance of white prepupal mRNA was designated 1 (15 μg per lane). Each bar indicates the mean ± S.E. of repeated experiments (n=4). 1L, 1st instar larva; 2L, 2nd instar larva; 3LE, early 3rd instar larva; 3LL, late 3rd instar larva; WP, white prepupa; BP, brown prepupa; 12P-36P, 12hr-36hr pupa; A, adult.

In order to measure the developmental changes of HSP23 content, Western blot analysis was performed (Fig. 3A). As similar to the developmental profiles of its mRNA, HSP23 content was detected for the first time in the early third instar larva (about 80 hrs after egg deposition). And then, the amount of HSP23 synthesis was increased as development proceeds, then peaked at the pupariation stage. After this stage, the amount of HSP23 was decreased with a slight fluctuation. The overall developmental profiles of HSP23 content and HSP23 mRNA were comparable.

Synthesis of ecdysteroids during development

To measure 20HE titer, ecdysteroids were isolated from the particular stages of whole organisms by methanol extraction and measured by RIA (Fig. 3B). The titers of ecdysteroids showed that there are considerable fluctuations as the development proceeds. The highest content of ecdysteroids was shown at the prepupal stage, and then sharply reduced as the development proceeds to adult. The developmental profile of ecdysteroids titer was consistent with the previous finding of Hodgetts *et al.* (1977) with a slight deviation. The deviation may be due to difference of antiserum used and synchronization of animals.

Relationship between HSP23 gene expression and 20HE synthesis

The effect of 20HE on HSP23 gene expression was investigated in the salivary glands *in vitro* (Fig. 4). The salivary glands were cultured in buffered saline and the newly synthesized proteins were labeled with [^{35}S] methionine. The synthesis of HSP23 was increased in a dose-dependent manner by addition of 20HE. The highest response was observed at the concentration of about 1-3 μmol of 20HE. At the dose more than 3 μmol, the induction of HSP23 synthesis was, however, reduced.

To determine whether HSP23 gene expression is regulated by 20HE *in vitro*, HSP23 and 20HE

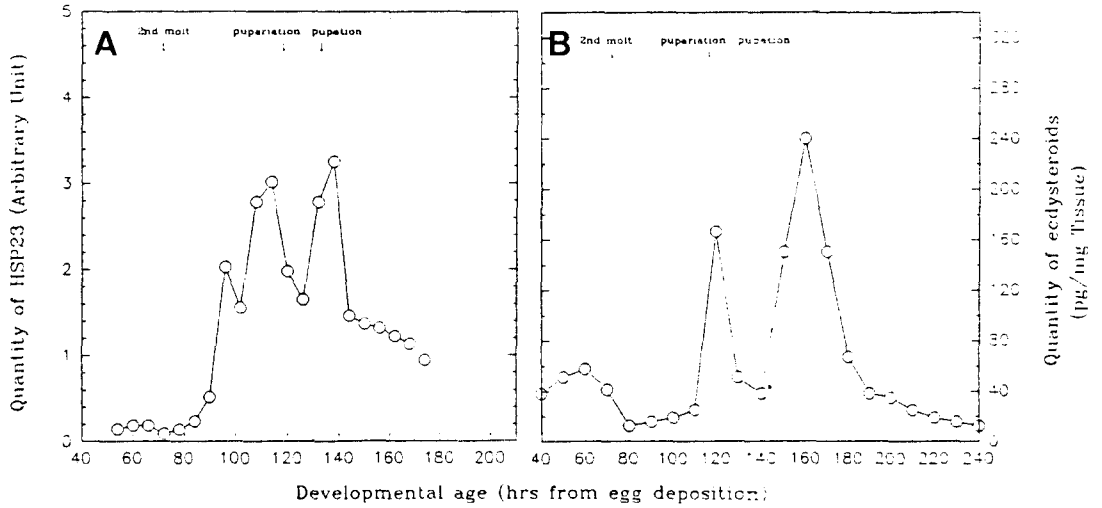
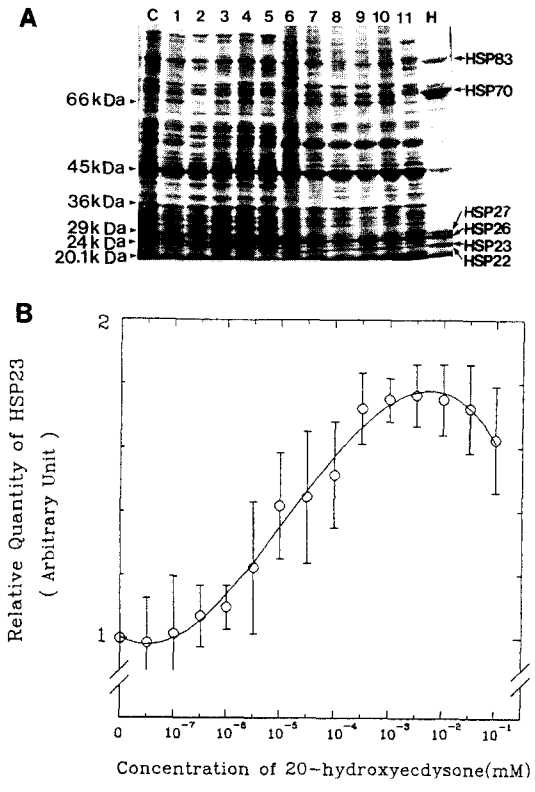


Fig. 3. Developmental profiles of HSP23 and ecdysteroids. (A) Developmental analysis of HSP23. The accumulating amounts of HSP23 were quantified by Western blot analysis using 1:250 dilution of anti-HSP23 antiserum and 1:500 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase. (B) Developmental analysis of ecdysteroids. The amounts of ecdysteroids were measured by RIA using dextran-coated charcoal method.



titers were compared during normal development. As shown in Fig. 3, there is a similarity between ecdysteroid titers and HSP23 gene expression during the third-instar larval-prepupal period. The patterns were, however, different from each other at the other developmental stages. For examples, at the first molting, 20HE showed a peak, but HSP23 was not detected. Also, titers of 20HE was still considerably high until the middle pupal period, but HSP23 was slightly synthesized at this time. Thus, it appears that HSP23 gene expression is controlled by endogenous 20HE only at the larva-pupa molting period. The stage-dependent control of HSP23 gene expression by

Fig. 4. Effect of 20HE on HSP23 gene expression in the cultured salivary glands. The salivary glands dissected from late third instar larvae were cultured in buffered saline containing various concentrations of 20HE. Proteins were labeled with [^{35}S] methionine and electrophoresed on the SDS-polyacrylamide gel (7-14% gradient). (A) Autoradiogram of electrophoresed gel. C, control; 1-11 lane, 20HE treatments at concentration of 0.1 nM, 1 nM, 10nM, 31.5 nM, 0.1 μM, 0.315 μM, 1 μM, 3.15 μM, 10 μM, 31.5 μM and 100 μM, respectively; H, heat shock at 37°C. (B) Quantification of HSP23 either by measuring the radioactivity of each HSP23 bands or by densitometrically. The equivalent of HSP23 amount of control was designated 1.

20HE provides a strong support for the notion that multiple regulatory controls are involved in the expression of heat shock genes (Zimmerman *et al.*, 1983; Riddihough and Pelham, 1986; Mestril *et al.*, 1986). Riddihough and Pelham (1986) and Mestril *et al.* (1986) reported that activation of *Drosophila* HSP27 and HSP23 genes by heat shock and ecdysone involves in the independent and remote regulatory sequences. Riddihough and Pelham (1987) showed that a 23-bp hyphenated dyad within ecdysone response element appears to be a protein-binding site. This sequence shows a partial homology with mammalian steroid receptor binding sites (Riddihough and Pelham, 1987).

Pelham (1986) speculated that some heat shock proteins play a role in protein folding and assembly which occur in cellular compartments. Upon heat shock or other stress, they may help to reassemble the disrupted cellular structures. The association of steroid receptors with heat shock proteins could be evident that 20HE controls the metamorphosis of larva into pupa through HSPs by operation of cellular protein repair or damage control machinery (Catelli *et al.*, 1985; Sanchez *et al.*, 1986).

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초파리에서 HSP23 유전자발현과 20-Hydroxyecdysone 합성의 발생학적 변화

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초파리에서 정상 발생동안 HSP23의 합성과 생리적 기능이 20-hydroxyecdysone (20HE)과 어떤 연관을 가지는지를 조사하였다. HSP23 유전자의 발현을 Northern blotting과 Western blotting으로 조사하였고, 20HE의 분비 양상은 radioimmunoassay 법으로 조사하였다. HSP23은 3령기 초기부터 발현이 시작되어 pupariation 시기에 급격한 증가를 보인 후 감소하였다. HSP23과 20HE의 합성을 비교하였을 때, 3령기 초기 pupa 시기에 두 분자가 모두 합성의 최고치를 보이며 유사한 합성 양상을 보였지만 그 외 시기는 일치하지 않았다. 따라서 20HE는 larva에서 pupa로의 변태 시기에 HSP23 유전자의 발현을 조절하며, 또 HSP23을 이용하여 변태과정의 일부를 조절하는 것으로 보인다.