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노랑초파리의 *ultraspiracle* 유전자 산물은 발생과정에서 광범위하게 발현되는 전사조정인자이다

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The *ultraspiracle* Gene Product is a transcriptional regulatory factor in *Drosophila* broadly Expressed throughout the organism during Development

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ABSTRACT : *ultraspiracle*(*usp*) gene produt(Usp) is a member of the superfamily of steroid hormone receptors in *Drosophila melanogaster* which mediate the hormone action by heteromerization with ecdysone receptor(EcR). Based on the genetic and molecular characterization of *usp*, it has been proposed that Usp funtions in at least three significant developmental pathway : embryogenesis, eye morphogenesis, and female reproduction. The expression patterns of Usp were investigated in individual tissues from different developmental stages of *Drosophila*. Usp is localized in the nucleus with ubiquitous distribution throughout development. This result suggests that Usp is not a transcriptional regulatory factor modulating the various process involving hormone response, but also associated with female and male reproduction in development of *Drosophila*.

Key words : *ultraspiracle*, *Drosophila*, development, immunocytochemistry

INTRODUCTION

Steroid and other nuclear hormone receptors are a family of ligand-modulated transcription factors that regulate cell differentiation and development as well as homeostasis and reproduction (Segraves, 1991; Oro *et al.*, 1992a). In *Drosophila melanogaster*, the steroid hormone 20-hydroxyecdysone triggers the key regulatory cascades controlling the coordinated changes in the developmental pathway of both larval and imaginal tissues in molting and metamorphosis (Steel and Davey, 1985; Raddiford, 1985). These major biological actions are mediated by the ecdysone receptor(EcR), a member of the nuclear hormone receptor superfamily (Koelle *et al.*, 1991). The ability of EcR to bind to hormone and to interact with ecdysone response element in the genome depends on the heterodimerization with Usp, the product of the *ultraspiracle*(*usp*) gene locus (Yao *et al.*, 1992; Thomas *et al.*, 1993; Yao *et al.*, 1993). Usp is one of several orphan receptors in *Drosophila*, sharing significant homology with the mammalian retinoid X receptor (Henrich *et al.*, 1990; Oro *et al.*, 1990; Shea *et al.*, 1990). Both Usp and RXRs can form heterodimers with several veterbrate nuclear receptors to modulate their affinity for genomic response elements (Yu *et al.*, 1992; Kliewer *et al.*, 1992; Bugge *et al.*, 1992; Leid *et al.*, 1992; Zhang *et al.*, 1992; Yao *et al.*, 1992, 1993). Among the transcriptional regulators induced by 20-hydroxyecdysone, some are other members of the receptor superfamily (Segraves and Hogness, 1990; Lavorgna *et al.*, 1993; Stone Thummel, 1993), rasing the possibility that the ecdysone receptor complex undergoes later modifications that alter its activity (Richards, 1992). Like Usp, these are orphan receptors for which no known hormone ligand has been identified (see Segraves, 1991). However, ectopic expression of *usp+* during *Drosophila* embryogenesis evokes no mutant effects, implying that its function also requires the presence of a localized ligand (Oro *et al.*, 1992b).

Genetically, the *usp* locus is defined by three lethal mutations which disrupt the organization of the posterior tip of the larvae both zygotically and maternally; second instar *usp*/Y larvae derived from heterozygous *usp*/+ mothers possess an extra set of spiracles, whereas *usp*/Y embryos derived from females possessing a germline clone(*usp*/*usp*) exhibit a localized ventral defect in the ninth or posterior eighth abdominal segment (Perrimon *et al.*, 1985; Oro *et al.*, 1992b). Germ-line mutant clones reveal a required maternal function

for the completion of embryogenesis (Perrimon *et al.*, 1985; Oro *et al.*, 1992b). Usp have been implicated in the regulation of s15 chorion gene expression (Shea *et al.*, 1990; Khoury Christianosan *et al.*, 1992) and retinal morphogenesis and female reproduction (Oro *et al.*, 1992b).

The behavior of Usp in heterodimerization formation and DNA binding assay *in vitro*, together with the pleitropy of *usp* gene function, suggests that *usp* is a regulatory generic nuclear receptor partner whose functional specificities result from various nuclear receptor associations (Richards, 1992). In this paper, the cellular and subcellular expression patterns of Usp during the development were investigated. This study showed that Usp was broadly expressed in various tissues throughout development.

MATERIALS AND METHODS

Drosophila Strains

Flies were raised at 25℃ on standard medium containing cornmeal, sugar, yeast and agar. Canton-S stocks is the wild-type strain in our studies.

Western Blot Analysis

The monoclonal antibody (AB11) to Usp protein used in this study was kindly provided by D. L. King and F. C. Kafatos. Staged embryos were washed from food plates, dechorionated in 50% bleach, and washed again in phosphate-buffered saline prior to total protein extraction. Larval tissues and adult tissues were dissected in *Drosophila* Ringer's medium and washed with 0.9% NaCl, 0.1% Triton X-100. After homogenizing in craking buffer (0.125 M Tris /HCl, pH 6.8, 5% mercaptoethanol, 0.1% Triton X-100, 4 M urea, 1 mM PMSF), supernatants were collected by centrifuging at 14,000 x g for 30 min. After protein contents were determined by Bio-Rad protein Assay kit, SDS was added to be 2% of final concentration in protein samples. After incubation for 5 min at 95℃, same amount of protien was applied to each slot on 12% SDS-polyacryamide gel. Following SDS-PAGE, protein was blotted to ProtoBlott™ membrane (Applied Biosystems). After blocking nonspecific protein binding by treatment with 5% non-fat dry milk in TBS (25 mM Tris/HCl, 0.5 M NaCl, pH 7.5) for 1 hr, blots were incubated for 2 hr at room temperature with AB11 monoclonal antibody (1 : 10 dilution of hybridoma supernatant in TBS). After three 15 min washes in TNT (TBS containing 0.1% Triton X-100), blots were incubated for 1 hr in peroxidase conjugated anti-mouse IgG

(Sigma) diluted 1 : 1,000 with TBS. Blots were washed in TNT three times for 15 min each, stained with DAB-H_2O_2 solution (0.001% 3, 3′-diaminobenzidine and 0.01% H_2O_2 in TBS).

Immunohistochemistry

Preparation and whole-mount antibody staining of embryos was carried out as described by Bopp *et al.* (1991) with some modification. Embryos were dechorionated for 3-5 min in 50% bleach, washed throughly in PBS, and incubated in haptane-saturated fixative (4% paraformaldehyde in PBS) for 30 min at room temperature. The fixative phase was then removed and replaced with 90% methanol, 10% EGTA [ethylene-glycol-bis (2-aminoethylester) - N, N - tetraacetic acid). After several vigorous shakes, the devitellinized embryos that sank to the bottom were collected and rinsed several times in the methanol-EGTA solution followed by rinse in plain methanol. For staining, embryos were gradually rehydrated in PBS and incubated with 1% BSA, 1% Triton X-100, in PBS, for 3-4 hr at room temperature. First antibody was applied as a 1 : 10 dilution of hybridoma supernatant (AB11) in PBS, 0.1% BSA, and 0.1% Triton X-100, and incubated overnight at 4℃. After several washes in the same buffer, embryos were treated with biotinylated second antibody for 2 hr at room temperature and subsequently with biotinylated HRP-avidin complexes according to the Vectastain protocol (Vector Laboratories). The bound complexes were visualized with a DAB solution (0.05% 3, 3′-diaminobenzidine and 0.025% H_2O_2 in PBS). I followed the ovary whole mount staining procedure of Suter and Steward (1991) to stain larval and adult tissues. The tissues were dissected in *Drosophila* Ringer's medium and fixed in 4% paraformaldehyde, PBS, for 20 min at room temperature. Tissues were permeabilized and blocked by incubating 1% BSA, 1% Triton X-100, and PBS for 4 hr at room temperature. Peroxidase staining and microscopy proceeded as described for embryos (see above).

In situ Hybridizations

In situ hybridization was carried out according to the method of Tautz and Pfeifle (1989). The embryos are collected, dechorionated, and fixed as described for immunocytochemistry. The ovary and larval tissues were dissected in *Drosophila* Ringer's medium and fixed in 4% paraformaldehyde. The embryo are first washed 3 times for 5 min each in PBT (PBS plus 0.1% Tween 20). They are then incubated for 3-5 min in 50 μg/ml Proteinase K in PBS. The digestion is stopped by incubating for 2 min

in 2 mg/ml glycine in PBT and the embryos are then washed 2 times for 5 min each in PBT, refixed for 20 min with 4% paraformaldehyde and finally washed 3 times for 10 min each in PBT. Digoxogenin-labeled antisense *usp* RNA probe was prepared with Boehringer Mannheim kit according to manufacturer's protocol. The hybridization solution consists of 50% formamide, 5 x SSC(1 x SSC is 150 mM NaCl, 15 mM sodium-citrate), 50 μg/ml heparin, 0.1% Tween 20 and 100 μg/ml sonicated and denatured salmon sperm DNA. Detection of hybridization was by an alkaline phosphatase conjugated anti-digoxigenin antibody. Hybridizations were carried out at 45℃ for 16 hours.

RESULTS

Genetic data have demonstrated clear requirement for *usp*+ function in embryogenesis, larval/pupal development and eye morphogenesis and female reproduction (Oro *et al.*, 1992b). The developmental profile of *usp* transcripts shows it to be expressed throughout the life cycle of the fly, consistent with the gene's multiple roles in development(Henrich *et al.*, 1994). Western blots using equal amounts of protein per lane showed that all tissues examined expressed Usp protein in unequal amounts(Fig. 1). The highest level of Usp expression was detected in imaginal disc and salivary gland from late third instar

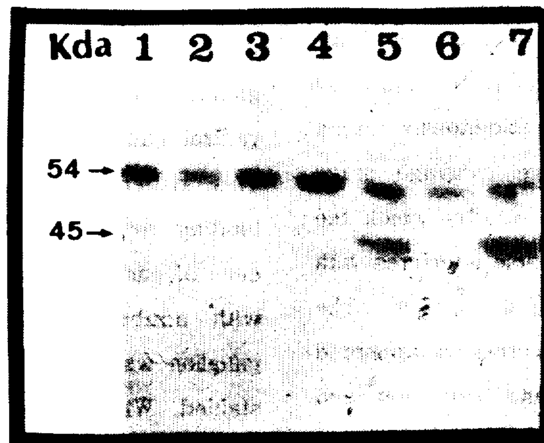

Fig. 1. Western blot analysis of indicated tissue extracts from late third instar larva and adults using anti-Usp monoclonal antibody(AB11). Lane 1, salivary glands from late 3rd instar larva; 2, fat bodies from late 3rd instar larva; 3, wing imaginal discs from late 3rd instar larva; 4, brain and ventral ganglions from late 3rd instar larva; 5, ovaries from mature females; 6, reproductive organs from mature males; 7, early embryos. Molecular weights x 10^{-3} are shown on the left.

larva, and the lowest level of Usp was detected in larval fat bodies and male reproductive organ. In early embryo extract and mature ovary extract, in addition to major Usp(54Kd), minor Usp having lower molecular weight 45 Kd was detected. At present it is not known whether this minor band is a degredation product or another isoform. To examine more presicely the temporal and spatial distribution of Usp, the ant-Usp monoclonal antibody(AB11) was used to stain embryo, larval tissues, and adult reproductive organs *in situ*.

The Expression of *usp* in Embryogenesis

Usp was uniquely localized to the nucleus of embryo, consisting with its roles as a member of nuclear hormone receptor superfamily of DNA binding proteins(Fig. 2). Usp protein was found to be expressed continuously and nearly ubiquitously during embryogenesis, from late cleavage stage (stage 3) through stage 16(after which the impermeability of the curticle interferes with whole mount staining(Fig. 2A-D). The highest levels of Usp expression appeared at the time of germband extension and these levels were uniformly high throughout the embryo. At later stages, Usp levels were highest in central nervous system. The germline primodium, the pole cells, were the one cell type that exhibited conspicuously low but significant Usp staining as these cells were transported to the center of the embryo at gastrulation. The Usp protein is also distributed throughout the embryo at all other stage that we have examined(data not shown). The *usp* RNA is homogeneously distributed throughout the whole embryo in early stage(Fig. 2E, F, G, H). After then, *usp* RNA accumulates uniformly in each cell with no apparent difference throughout embryogensis as shown in protein staining.

The Expression of *usp* in Larval Tissues

Usp protein was also detected during post-embryonic development(Fig. 3). In late third instar larva, the highest level of Usp was found in imiginal discs(eye-antenna, wing, leg), ring gland, and salivary glands. While fat bodies and brain and ventral ganglion show relatively low levels. This pattern is consistent with Western blotting results(Fig. 1). Essentially all of cells of each tissues appeared to be stained with antibody except brain and ventral ganglion which were far less homogeneously stained. While nearly all of the cells in the proliferation zone of the cerebral hemispheres were heavily stained, only a fraction of ventral ganglion were found to contain Usp(Fig. 3E, F, G, H), and these Usp-containing cells have not been char-

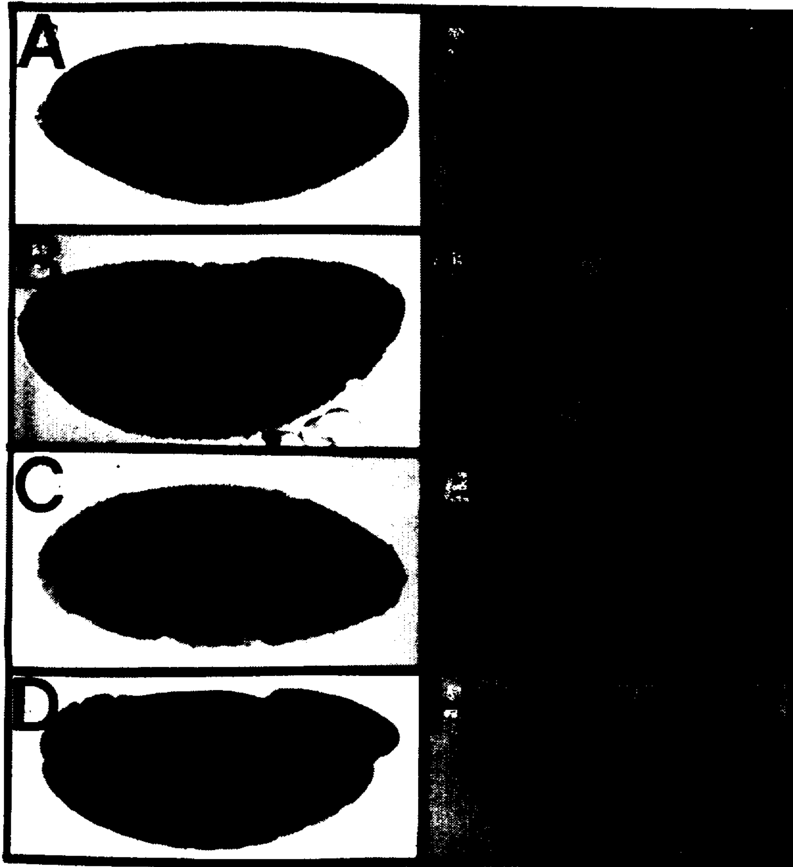

Fig. 2. Expression of Usp and ***usp*** RNA throughout embryonic development. Wild-type embryos were labeled with anti-Usp monoclonal antibody (AB11) and antisense ***usp*** RNA probe. (A) Late cleavage stage embryo showing dark staining in the syncytial nuclei (situated around the periphery of the embryo) and light staining in the newly formed pole cells. (B) Blastoderm/early gastrula embryo. (C) Extanded germ band/gastrulating embryo. (D) Retracted germ band/late stage embryo showing widespread expression Usp protein. At this stage there is particularly intense staining in the central nervous system. Negative controls consisting of control culture medium containing mouse IgG (20 μg/ml) or without first antibody showed no background staining (data not shown). (E, F, G, H) The spatial distribution of ***usp*** RNA using non-radioactive detection in same stage embryo shown in left panel. ***usp*** RNA accumulates uniformly in each cell with no apparent difference. Low levels of specific ***usp*** staining were observed that were not seen with control probe. All embryo are oriented with anterior to the right and dorsal approximately up.

Fig. 3. Expression of Usp and *usp* RNA in larval tissues. Tissues from wild-type larvae were labeled with anti-Usp monoclonal antibodoy(AB11) and antisense *usp* RNA probe. (A, B, C) Salivary gland from 2nd insta, early 3rd instar, wandering 3rd instar larvae, respectively. (D) Ring gland from wandering 3rd instar larvae. (E, F, G, H) Brain and ventral ganglion from 2nd instar, early 3rd instar, wandering 3rd instar, puparium formation, respectively. (I, J, K) Wing imaginal disc, eye/antennal imaginal disc, leg disc from wandering 3rd instar larvae, respectively. (L, M, N) The spatial distribution of *usp* RNA using non-radioactive detection in wing disc, eye/antennal disc, and leg imaginal disc, respectively. *usp* RNA accumulates uniformly in each cell with no apparent difference. Low levels of specific *usp* staining were observed that were not seen with control probe.

acterized further. In addition, we have seen nuclear staining in all other tissues eximined, gut, trachea, and cells associated with cuticular structure(data not shown). Also, *usp* RNA accumulates uniformly in each cell of wing disc, eye antennal disc, and leg disc(Fig. 3L, M, N).

The Expression of *usp* in Adult Reproductive Tissues

Usp protein functions in female reproduction (Oro *et al.*, 1992b) and choriongenesis (Shea *et al.*, 1990). The present study extends the genetic findings by examining the expression pattern of *usp* in ovary. Usp staining is continuosly seen in both the germ line cells and follicle cells from germarium stage to the differentiated egg chambers(Fig. 4). At early stage of oogenesis, Usp is localized to the nucleus and expressed at high level in follicle cells

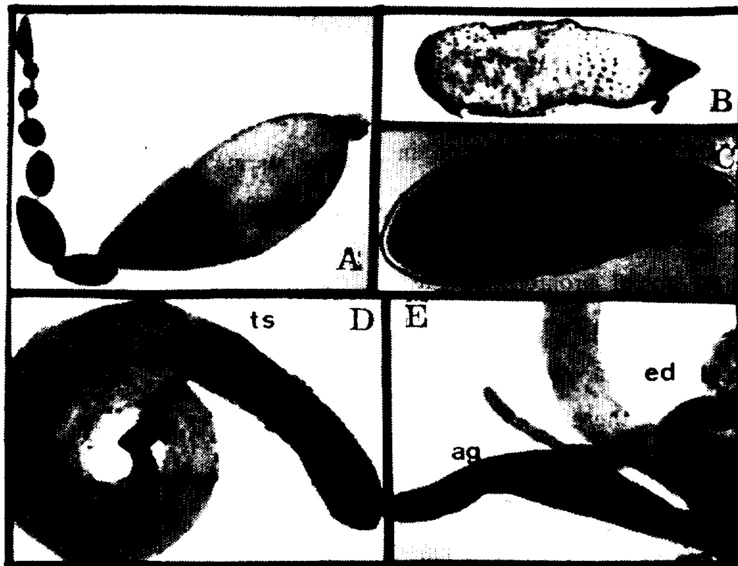

Fig. 4. Expression of Usp and *usp* RNA in developing egg chambers and male reproductive organs. The tissues dissected from wild-type adult flies were labeled with anti-Usp monoclonal antibody(AB11) and antisense *usp* RNA. (A) Usp expression in wild-type ovary. Usp is expressed in both germ cells and follicle cells of all stage of egg chambers. (B) Usp expression in stage 11 egg chambers. Note the apparent Usp expression in both degenerating nurse cells and follicle cells. (C) The spatial distribution of *usp* RNA using non-radioactive detection in egg chambers. The patterns of *usp* RNA accumulations are similar to those of Usp expression. Low levels of specific *usp* staining were observed that were not seen with control probe. (D, E) Expression of Usp in male reproductive organs. Nuclear staining is apparent in the tip and somatic cells of testis(ts), and in ejaculatory duct(ed) and accessory gland(ac). Negative controls prepared with control culture medium containing mouse IgG (20 μg/ml) or without first antibody showed no backgroud staining(data not shown).

and at relatively low level in germline cells (Fig. 4A). At the begining of vitellogenesis (stage 7), Usp is expressed at a high level in nurse cells. At stage 11 egg chamber, Usp expression remained continuously both in degenerating nurse cells and follicle epithelium, which become thinner and starts secreting the endochorion (Fig. 4B). The patterns of *usp* RNA accumulations are similar to those of Usp expression in developing egg chambers (Fig. 4C).

Although no requirement for *usp+* during spermatogenesis has been recognized genetically, we did find Usp expression in adult male gonads (Fig. 4D, E). There was dark nuclear staining at the tip of the testis, a region of gonial proliferation (Fig. 4D). Throughout the rest of the testis, somatic cyst cell nuclei were heavily stained. In addition, there was intense staining in the seminal vesicle and ejaculatory duct epithelia, and accessory gland (paragonia) epithelia of newly eclosed male (Fig. 4E).

DISCUSSION

Genetic and molecular analyses of *usp* suggest that Usp is continuously required throughout development. We showed previously that the Usp expression was not confined to developmental periods and cell-types associated specifically with major ecdysone-inducible events, rather it undergoes numerous and frequent changes during embryonic, larval, and adult development (Henrich *et al.*, 1994). In this study the temporal and spatial distribution of Usp in embryos, larval tissues, and adult reproductive organs were examined.

Usp is a nuclear partner of EcR *in vivo*

In *Drosophila* development, at least six pulses of ecdysone are thought to occur, one during each stage of development : embryonic, three larval instar, prepupal and pupal (Richard, 1981). So, diverse response to ecdysone occur at different developmental stages, and within a stage, in different tissues. Usp are expressed nearly in all cells throughout embryogenesis and larval development. EcR is also expressed at high levels in these developmental stages (Koelle *et al.*, 1991). It seems that the major hormone-inducible events is initiated by heteromerization Usp and EcR *in vivo* during these developmental stages (Yao *et al.*, 1993), though the possiblilty could not be excluded such that the other orphan receptors replace the Usp's role (Richards, 1992). Also the ectopic expression of *usp+* during embryogenesis evokes no mutant effects, implying that its function also requires the

presence of a localized ligand (Oro *et al.*, 1992b). Ecdysteroids produced in *Drosophila* females may be sequestered in oocytes, as they are in other insects (Sall *et al.*, 1983; Isaac and Rees, 1985; Dubendorfer and Maroy, 1986). The ecdysteroids deficiency in embryos using *ecdysoneless(ecd)* mutant cause embryonic lethality (Henrich *et al.*, 1994). *Drosophila* females reared on sterol mutants of the yeast *Saccharomyces cerevisiae* produced less eggs than flies reared on wild type yeast and the few eggs laid had a reduced egg hatchability (Bos *et al.*, 1976). These data indicate, that ecdysterids of maternal origin would be essential for the embryogenesis.

Virtually all tissues exhibit some kind of morphological changes in response to the metamorphic ecdysone pulse that peaks at the end of larval life (Bodentein, 1950). During metamorphosis, larval tissues are stimulated to histolyse and imaginal tissues to begin their differentiation to adult structure. According to Talbot *et al.* (1993), at the onset of metamorphosis different ecdysone target tissues express different EcR isoform combination in a manner consistent with the proposition that the different metamorphic response of these tissues require different combination of the EcR isoforms. Usp protein is expressed in all ecdysone target tissues during these developmental stage, allowing the heterodimerization with EcR to initiate the hormone response. The analysis of *usp* mitotic clones suggests that wild-type *usp* function is not required for the development of the adult cuticular structures of the notum and abdomen (Oro *et al.*, 1992b). Other members of the steroid receptor superfamily, many of which have been identified (Segraves, 1991), might be able to substitute for Usp as pairing partners for the EcR proteins (Talbot *et al.*, 1993). This results showed the Usp was expressed in the wing imaginal discs, suggesting some role for *usp* in normal morphogenesis of wing imaginal discs.

Usp seems to be involved in both female and male reproduction

Using the conditional expression system coupled with mosaic analysis, Oro *et al.* (1992b) suggested that *usp* function in egg shell synthesis and female fertility. Maternal somatic *usp* function is required for fertilization of the egg, maternal germline *usp* function is required for a late embryonic functions and choriogenesis (Oro *et al.*, 1992b). They showed also that ovarian *usp* transcript was expressed predominantly in the nurse cells and not in follicle cells up to stage 12 of oogenesis. In contrast, Shea *et al.* (1990) identified *usp* cDNA from follicle-cell-enriched expression library on the basis of its ability to bind chorion s15 promoter.

Also, Usp immunostaining revealed the existence of the Usp protein in germ-line nurse cells as well as follicle cells (Khoury Christianson *et al.*, 1992; this paper). Ecdysteriods are present in adult *Drosophila* (Hodgetts *et al.*, 1977; Grau and Lafont, 1994), and in many insects they have important rules in reproduction. *Drosophila* ovaries synthesize and secrete ecdysteroid *in vitro* (Rubenstein *et al.*, 1982). they can stimulate yolk protein synthesis, individually or together with juvenile hormones (Postlethwait and Jowett, 1981). Considering this data, Usp is not only required for fertilization of the egg and choriogenesis, but also needed for ovarian differentiation.

Usp protein is present in male reproductive tissues where there is no known function for gene. Usp staining is detected in the cells at the tip of the testis from newly eclosed and matured male. Usp is also expressed at high levels in the undifferentiated accessory gland, seminal vesicle, and ejaculatory duct from newly eclosed males, suggesting some Usp's role in the terminal differentiation of these reproductive tracts. Ecdysteriod titres in whole flies decreased as the flies matured after eclosion (Bownes *et al.*, 1984), and there is a juvenile hormone peak at eclosion (Bownes and Rembold, 1987). The accessory gland synthesis and secretes a complex mix of proteins and peptides that repress female sexual receptivity and stimulate oviposition (Chen, 1984; DiBenedetto *et al.*, 1987; Chen *et al.*, 1988). The glucose dehydrogenase (GLD), which gene expression is correlated with ecdysone titer (Cavener *et al.*, 1986) is expressed in both sexes during metamorphosis, but shortly after eclosion GLD activity rises in a male ejaculatory duct. Considering these observation, Usp may play some role in modulating the gene expression associated with male reproduction.

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적 요

노랑초파리의 *ultraspiracle* 유전자 산물 (Usp)은 ecdysone receptor(EcR)와 이합체를 형성함으로서 호르몬의 작용을 매개하는 전사인자로써 스테로이드 호르몬 수용체군에 속한다. 유전적인 분석과 분자생물학적 분석을 의하면 Usp는 초파리 배발생, 눈의 형태형성, 암컷의 생식과정에 관여한다. 본 연구에서는 발생과정중에 각 조직에서 *usp* 유전자의 발현 양상을 immunocytochemistry와 in situ hybridization 방법으로 조사하였다. Usp의 발현은 발생의 전과정을 통해 조사된 모든 조직의 핵에서 검출되었다. 본 연구결과는 Usp는 발생과정에서 호르몬의 작용을 매개할 뿐 아니라 암컷과 수컷의 생식과정에도 관여한다고 사료된다.