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The Expression of *ultraspiracle* Gene Product during Development of *Drosophila melanogaster*

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***ultraspiracle* (*usp*) gene product (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. In this study, the expression patterns of Usp were investigated by immunohistochemistry in individual tissues from differnt developmental stages of *Drosophila*. Usp is localized in the nucleus with ubiquitous distribution throughout development. Usp expression is detected throughout embryogenesis. Usp is expressed in imaginal and larval tissues from late third instar larva. The expression pattern of Usp is overlapped by those of EcR. Also Usp is expressed in differentiating adult reproductive organs. This result suggests that Usp is not a transcriptional regulatory factor modulating hormonal response during development, but also play some roles in female and male reproduction of *Drosophila*.**

KEY WORDS: *ultraspiracle*, *Drosophila*, Development, Immunohistochemistry

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 vertebrate nuclear receptors to modulate their affinity for genomic response elements (Yu *et al.*, 1991; 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 and 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 (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, we describe the cellular and subcellular expression patterns of Usp during the development. We show that Usp is broadly expressed in various tissues throughout development.

Materials and Methods

Drosophila Strains

Flies were raised at 25°C on standard medium containing cornmeal, sugar, yeast and agar. Canton-S stock is the wild-type strain used in this study.

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 ×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° C, same amount of protein was applied to each slot on 12% SDS-polyacrylamide gel. Following SDS-PAGE, protein was blotted to ProtoBlott^{TM} 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 for overnight at 4°C. 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). For confocal microscopy (Zeiss LSM410 system), we used FITC-conjugated secondary antibody. Confocal images were produced using Montage FR2 film recorder with Kodak T-Max 100 film. We 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 in 1% BSA, 1% Triton X-100, and PBS for 4 hr at room temperature. Peroxidase staining, fluorescence staining, and microscopy proceeded as described for embryos (see above).

Results and Discussion

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. And similarly, 54 Kd Usp protein is present throughout development and in adults, but not at the same quantitative level at each of these stages (Henrich *et al.*, 1994a). In this study we examined the temporal and spatial distribution of Usp in embryos, larval tissues, and adult reproductive organs.

Western blots using equal amounts of protein per lane showed that Usp protein was expressed in all tissues examined (Fig. 1). The highest level of Usp expression was detected in imaginal disc and salivary gland from late third instar 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 (54 Kd), minor Usp having lower molecular weight 45 Kd was detected. At present it is not know whether this minor band is a degredation product or another isoform. To examine more presicely the temporal and spatial distribution of Usp, the anti-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. 2A). Usp protein was found to be expressed continuously and nearly ubiquitously during embryogenesis, from late cleavage stage (stage 3) through stage 16 (after

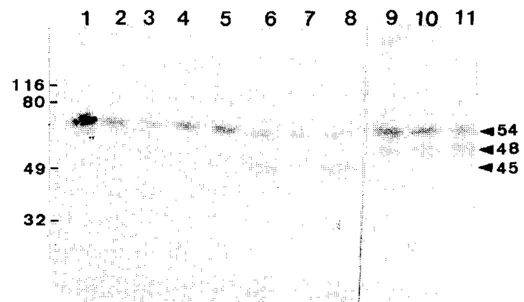

Fig. 1. Western blot analysis of indicated tissue extracts from late third instar larva and adults using anti-USP monoclonal antibody (AB11). Lane 1, Usp produced from baculovirus as positive control; 2, salivary glands from late 3rd instar larva; 3, fat bodies from late 3rd instar larva; 4, wing imaginal discs from late 3rd instar larva; 5, brain and ventral ganglions from late 3rd instar larva; 6, ovaries from mature females; 7, reproductive organs from mature males; 8, early embryos (0-3hrs); 9, 10, 11, whole animal extracts from pupa, newly eclosed male, and newly eclosed female, respectively. Molecular weights x 10^{-3} are shown on the left.

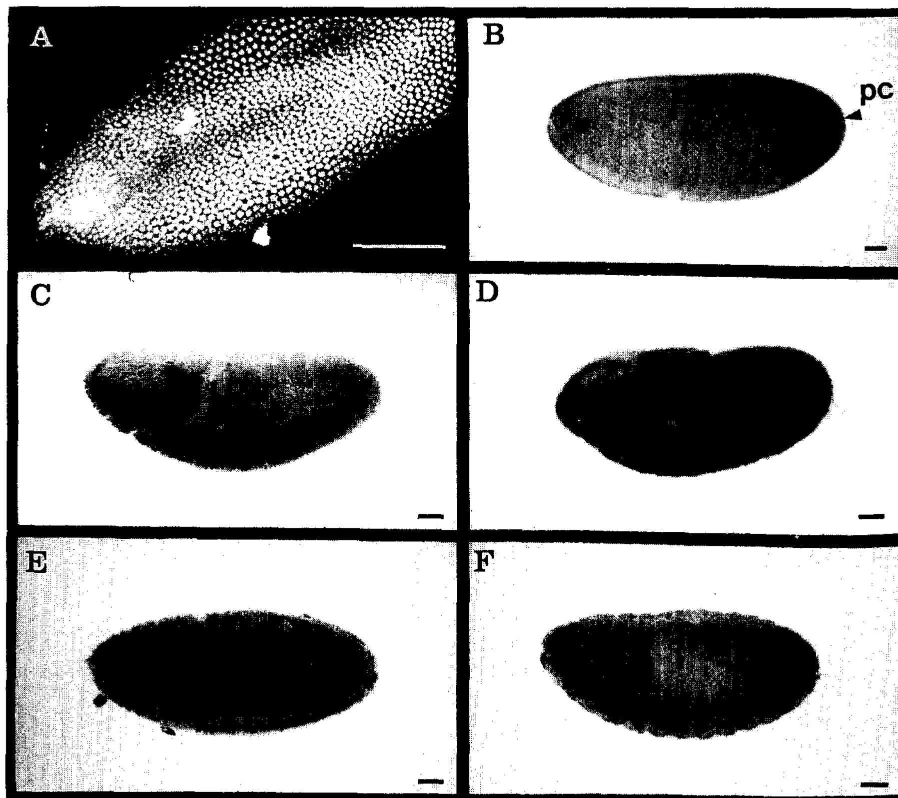

Fig. 2. Expression of Usp throughout embryonic development. Wild-type embryos were labeled with anti-Usp monoclonal antibody (AB11). (A) Nuclear localization of Usp in cellular blastoderm stage embryo shown by confocal microscopy. (B) Late cleavage stage embryo showing dark staining in the syncytial nuclei (situated around the periphery of the embryo) and light staining in the newly formed pole cells (pc). (C) Blastoderm/early gastrula embryo. (D) Extanded germ band/gastrulating embryo. (E, F) Retracted germ band/late stage embryo showing widespread expression Usp protein. At this stage there is particularly intense staining in the central nerves 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). All embryo are oriented with anterior to the left and dorsal approximately up. Bar indicates a length of 50 μm.

which the impermeability of the curticle interferes with whole mount staining (Fig. 2B-H). 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).

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). 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.*, 1994b). *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. Usp are expressed nearly in all cells throughout embryogenesis. EcR is also expressed at high levels in these developmental stages (Koelle *et al.*, 1991). It seems that the major ecdysone-inducible events are initiated by heteromerization of Usp and EcR *in vivo* during embryogensis, though the possiblilty could not be excluded such that the other orphan receptors replace the Usp's role (Richards, 1992) or Usp's function requires the presence of a localized ligand (Oro *et al.*, 1992b).

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. Also Usp was detected in peripheral glias (Fig. 3F). In addition, we have seen nuclear staining in all other tissues examined, gut, trachea, and cells associated with cuticular structure (data not shown).

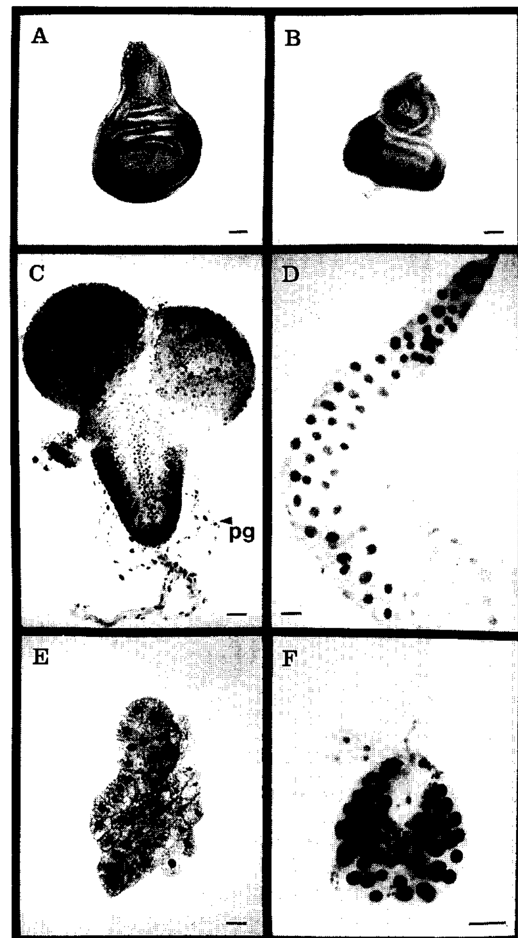

Fig. 3. Expression of Usp in larval tissues. Tissues from wild-type late 3rd instar larvae were labeled with anti-Usp monoclonal antibodoy (AB11). (A) Wing disc. (B) Eye/antennal imaginal disc. (C) Brain and ventral ganglion. m, mushroom body neuron; o, optical lobe; pc, peripheral glia. (D) Salivary gland. (E) Fat body. (F) Ring gland. Negative controls consisting of control culture medium containing mouse IgG (20 μg/ml) or without first antibody showed no background staining (data not shown). Bar indicates a length of 50 μm.

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. At the onset of metamorphosis different ecdysone target tissues express different EcR isoform (EcR-A, EcR-B1, and EcR-B2) combination in a manner consistent with the proposition that the different metamorphic response of these tissues require different combination of the EcR isoforms (Talbot *et al.*, 1993; Truman *et al.*, 1994). Usp is expressed in all ecdysone target tissues during these developmental stage, allowing the heterodimerization with EcR isoforms 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). By contrast, examination of transheterozygous for *usp*$^-$ and *EcR*$^-$ null mutations shows that they exhibit defective wing phenotypes, suggesting that both *usp* and *EcR* functions are required for normal metamorphosis of wing imaginal disc, which also yields the notum (Henrich, unpublished). Our results showed the Usp is expressed in the wing imaginal discs, suggesting some role for *usp* in normal morphogenesis of wing imaginal discs.

The Expression of Usp in Adult Reproductive Tissues

Usp 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 of 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 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. 4D).

Although no requirement for *usp*$^+$ during spermatogenesis has been recognized genetically, we did find Usp expression in adult male gonads (Fig. 4E, F). There was dark nuclear staining at the tip of the testis, a region of gonial proliferation (Fig. 4F). 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).

Using the conditional expression system coupled with mosaic analysis, Oro *et al.* (1992b) showed 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 is expressed predominantly in the nurse cells and not in follicle cells up to stage 12 of oogenesis. Oro *et al.* (1992b) suggest that the effects of *usp* on choriogenesis is one in which normal choriogenesis requires *usp*-dependent local signalling between the germline and soma. 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 roles 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. It is possible that Usp's role in these tissues is redundant, i.e. nonessential, such that specific phenotypes have not been identified with *usp* mutations. Or, extant *usp* mutant alleles might not allow the recognition of some essential functions. For example, the death of individuals homozygous for an *usp* allele would obscure any *usp*$^+$ role in male reproduction. 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 roles in the terminal differentiation of these reproductive tracts. Ecdysteriod titres in whole flies decreased as the flies matured after eclosion (Bownes *et al.*, 1984),

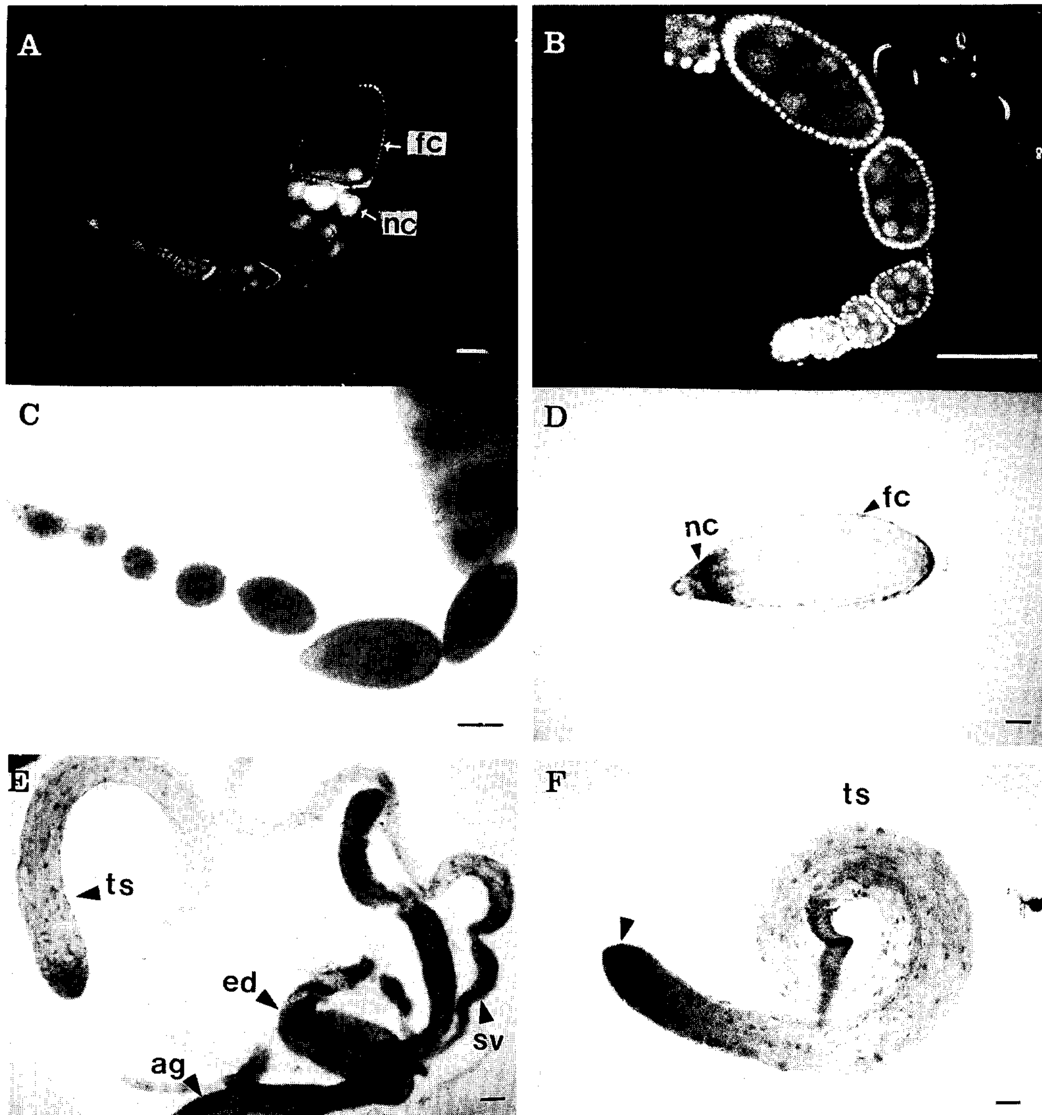

Fig. 4. Expression of Usp in female developing egg chambers and male reproductive organs. Reproductive organs were dessected from wild-type adult female and males and labeled with anti-Usp monoclonal antibody (AB11). (A, B) Usp expression in wild-type ovary shown by confocal microscopy. Usp is expressed in both germ cells and follicle cells of all stage of egg chamber. (C, D) Usp expression in early stages and stage 11 egg chambers using peroxidase staining, respectively. Note the apparent Usp expression in both degenerating nurse cells (nc) and follicle cells (fc). (E) Newly eclosed male reproductive organ. Nuclear staining is apparent in the tip and somatic cells of testis (ts). Usp expression is detected in epithelial cells of the seminal vesicle (s) and ejaculatory duct (ed) and accessory gland (ac). (F) Usp expression in testis (ts) from mature male. Usp staining is strong in the cells at the tip of the testis (arrow head). Negative controls prepared with control culture medium containing mouse IgG (20 μg/ml) or without first antibody showed no background staining(data not shown). Bar indicates a length of 50 μm.

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. However we can't exclude the possibility that Usp's function are dependent on localized ligand, since ectopic expression of usp^+ during *Drosophila* embryogenesis evokes no mutant effects (Oro *et al.*, 1992b). Considering these observation, Usp may play some roles in modulating the gene expression associated with male reproduction.

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노랑초파리 발생과정에서의 *ultraspiracle* 유전자 산물의 발현

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노랑초파리의 *ultraspiracle*(*usp*) 유전자 산물(Usp)은 steroid hormone receptors의 일원으로서 ecdysone receptor(EcR)와 이합체를 형성하여 호르몬의 작용을 매개한다. usp에 대한 유전학적, 분자생물학적 분석을 통해 Usp는 적어도 3가지의 발생경로(embryogenesis, eye morphogenesis, female reproduction)에 관여한다고 알려지고 있다. 본 연구에서는 초파리의 발생과정에서의 usp의 발현 양상을 immunohistochemistry 방법으로 분석하였다. Usp의 발현양상은 EcR의 발현양상과 유사하였다. Usp는 배발생과정, 유충시기, 그리고 성체의 생식기관에서 발현되었고, 분석된 모든 조직에서 핵내에 위치함이 관찰되었다. 따라서 Usp는 발생과정중에 호르몬의 작용을 매개할 뿐만 아니라 female과 male의 생식기관의 분화에도 관여하는 전사조정 인자라고 사료된다.