Developmental Biology 328 (2009) 541–551

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

EcR-B1 and Usp nuclear hormone receptors regulate expression of the *VM32E* eggshell gene during *Drosophila* oogenesis

Fabio Bernardi [a,1], Patrizia Romani [a,1], George Tzertzinis [b], Giuseppe Gargiulo [a,*], Valeria Cavaliere [a,*]

[a] *Dipartimento di Biologia Evoluzionistica Sperimentale, Università di Bologna, Bologna, Italy*
[b] *RNA Biology Division New England Biolabs, Ipswich, MA, USA*

ARTICLE INFO

Article history:
Received for publication 29 July 2008
Revised 30 December 2008
Accepted 10 January 2009
Available online 20 January 2009

Keywords:
Drosophila
Ecdysone signaling
EcR
Usp
Eggshell
VM32E
VM26A.2

ABSTRACT

Ecdysone signaling plays key roles in *Drosophila* oogenesis, as its activity is required at multiple steps during egg chamber maturation. Recently, its involvement has been reported on eggshell production by controlling chorion gene transcription and amplification. Here, we present evidence that ecdysone signaling also controls the expression of the eggshell gene *VM32E*, whose product is a component of vitelline membrane and endochorion layers. Specifically blocking the function of the different Ecdysone receptor (EcR) isoforms we demonstrate that EcR-B1 is responsible for ecdysone-mediated *VM32E* transcriptional regulation. Moreover, we show that the EcR partner Ultraspiracle (Usp) is also necessary for *VM32E* expression. By analyzing the activity of specific *VM32E* regulatory regions in usp^2 clones we identify the promoter region mediating ecdysone-dependent *VM32E* expression. By *in vitro* binding assay and site-directed mutagenesis we demonstrate that this region contains a Usp binding site necessary for *VM32E* regulation.
Our results further support the crucial role of ecdysone signaling in controlling transcription of eggshell structural genes and suggest that the heterodimeric complex EcR-B1/Usp mediates the ecdysone-dependent *VM32E* transcriptional activation in the main body follicle cells.

Introduction

Hormonal signaling plays key regulatory roles in *Drosophila* development and principally involves the steroid 20-hydroxyecdysone (20E) (Henrich and Brown, 1995; Riddiford et al., 2000; Thummel and Chory, 2002). Ecdysone activity is mediated by a heterodimer of two nuclear receptors, Ecdysone receptor (EcR) and Ultraspiracle (Usp) (Yao et al., 1992, 1993; Thomas et al., 1993). The EcR/Usp heterodimer directly controls expression of early ecdysone response genes, which coordinate the subsequent transcription of tissue-specific late genes (Ashburner et al., 1974; Thummel, 1996). *EcR* encodes three protein isoforms: EcR-A, EcR-B1 and EcR-B2. These proteins have common DNA- and ligand-binding domains but differ in their N-terminal region, which is probably responsible for the response specificity to the ecdysone stimulus. Moreover the three isoforms have different spatial and temporal expression profiles (Talbot et al., 1993; Truman et al., 1994) triggering specific cellular responses to ecdysone stimuli during different developmental stages (Bender et al., 1997; Schubiger et al., 1998). The EcR partner Usp is the *Drosophila* RXR homologue (Oro et al., 1990). The EcR/Usp heterodimer controls gene transcription by binding to specific sequences named Ecdysone Response Elements (EcREs) (Riddihough and Pelham, 1987; Cherbas et al., 1990). Both *in vitro* and *in vivo* analyses have led to the identification of various EcREs, which are invariantly composed of a direct or inverted repeat of the consensus sequence PuG(G/T)TCA spaced by an unconserved sequence spanning from 1 to 12 nucleotides (Antoniewski et al., 1993, 1996; D'Avino et al., 1995).

Genetic, biochemical and physiological studies have established the importance of ecdysone signaling also in oogenesis. From these analyses a pleiotropic function has emerged. Indeed, ecdysone signaling controls production of yolk proteins (Bownes, 1982; Carney and Bender, 2000; Terashima and Bownes, 2004), egg chambers maturation (Buszczak et al., 1999; Gaziova et al., 2004; Soller et al., 1999), follicular epithelium morphogenesis (Romani et al., 2009), border cell migration (Cherbas et al., 2003; Bai et al., 2000; Hackney et al., 2007) and eggshell formation (Cherbas et al., 2003; Hackney et al., 2007; Oro et al., 1992; Tzolovsky et al., 1999). Among the observed phenotypes, inhibition of ecdysone signaling results in thin eggshell (Oro et al., 1992; Hackney et al., 2007), pointing to its role in controlling eggshell morphogenesis. As previously reported an EcRE is present in *s15* chorion gene promoter and the downstream half-site of this element is bound *in vitro* by Usp (Shea et al., 1990; Khoury-Christianson et al., 1992). Moreover Hackney et al. (2007) have recently demonstrated that EcR regulates chorion gene expression and amplification. In the present study we investigate whether ecdysone signaling plays a more general role in controlling eggshell

* Corresponding authors.
E-mail addresses: giuseppe.gargiulo@unibo.it (G. Gargiulo), valeria.cavaliere@unibo.it (V. Cavaliere).
[1] F. Bernardi and P. Romani contributed equally to this work.

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.01.013

assembly, regulating also transcription of the two best characterized vitelline membrane genes: *VM32E* and *VM26A.2*.

Time-coordinated expression of eggshell structural genes is one of the key factors in eggshell formation but little is known about the signaling pathways underlying this transcriptional regulation. *Drosophila* eggshell constituents are synthesized in the follicle cells starting at stage 8 in a well-defined spatial and temporal pattern that reflects their structural contribution to the different layers making up the eggshell. Between stages 8 and 10 vitelline membrane genes are expressed, while chorion gene transcription begins at stage 11 and proceeds until the end of oogenesis (reviewed in Cavaliere et al., 2008; Mahowald, 1972; Petri et al., 1976; Waring and Mahowald, 1979; Fargnoli and Waring, 1982). *VM32E* was assigned to the vitelline membrane gene family, based both on its stage-specific expression pattern and on the presence of a conserved aminoacid sequence called VM domain (Gigliotti et al., 1989). Beside these features, shared with the other best-characterized vitelline membrane gene *VM26A.2* (Burke et al., 1987; Popodi et al., 1988), *VM32E* presents some peculiarity. Its expression is restricted to stage 10 of oogenesis. At stage 10A *VM32E* mRNA begins to accumulate in the ventral domain of the follicular epithelium and subsequently it becomes detectable also on the dorsal side of the egg chamber. Moreover *VM32E* transcription is tightly regulated spatially. It is expressed in the main body follicle cells except for centripetal cells and the most posterior follicle cells (Gargiulo et al., 1991). This expression pattern depends on a complex transcriptional regulatory network. We have previously identified three main *cis*-acting regulatory regions in *VM32E* promoter controlling its expression in different follicle cell domains (Cavaliere et al., 1997; Andrenacci et al., 2000). More recently we demonstrated the contribution of Decapentaplegic (Dpp) and Epidermal Growth Factor Receptor (EGFR) pathways in *VM32E* transcriptional regulation: the former repressing *VM32E* expression in the centripetal cells (Bernardi et al., 2006) and the latter modulating it in the main body follicle cells (Bernardi et al., 2007).

Immunohistochemistry analyses have revealed other features characterizing *VM32E*. Although not expressed in the centripetal and most posterior follicle cells, once secreted VM32E is able to spread to the terminal domains, becoming uniformly distributed around the oocyte at stage 11. In addition, in late stage egg chambers VM32E is present in the vitelline membrane and also in the endochorion, suggesting it contributes to the assembly of both eggshell layers (Andrenacci et al., 2001).

In the present study we show that *VM32E* but not *VM26A.2* expression is dependent on ecdysone signaling and this control requires Usp and EcR-B1 nuclear receptors. Therefore, this signaling pathway does not appear to play a general role for the activation of the other members of the vitelline membrane gene family. These results highlight the distinguishing characteristics of *VM32E* gene, which shares common features with both vitelline membrane and chorion genes and confirm the key role played by ecdysone in eggshell assembly.

Materials and methods

Fly strains

Stocks were raised on standard cornmeal/yeast/agar medium at 25 °C, and crosses were made at the same temperature unless otherwise stated. *y, w*^{67c23} was used as the wild-type stock in this study. Stocks used for clonal analysis were *P{ry*$^{+}$*, hsFlp}, y*^{1}*, w*^{1118}*; Dr*^{Mio}*/TM3, ry, Sb*^{1} (Bloomington stock 7), *P{ry*$^{+t7.2}$ *= hsFLP}1, w*^{1118}*; Adv*^{1}*/CyO* (Bloomington stock 6), *y*^{1}*, w*, P{w*$^{+mC}$ *= GAL4-Act5C(FRT.CD2).P}D* (Bloomington stock 4779), *w*^{1118}; P{*w*$^{+mc}$ = UAS-EcR-RNAi}104 (Bloomington stock 9327), *w*^{1118}*; P{UAS-EcR.A.dsRNA}91/TM3, P{ActGFP}JMR2, Ser*^{1} (Bloomington stock 9328), *w*^{1118}*; P{w*$^{+mC}$ *= UAS-EcR.B1.dsRNA}168* (Bloomington stock 9329), w*; P{w^{+mC} = UAS-EcR.B2.F645A}TP1 (Bloomington stock 9450), *P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w*; P{UAS-mCD8::GFP.L}LL5* (Bloomington stock 5134), *w*^{1118}*, P{Ubi-GFP(S65T)nls}X, P{neoFRT}18A* (Bloomington stock 5623), *y*^{1}*, w**; P{*w*$^{+mC}$ = *tub*P-GAL4}LL7/TM3, *Sb*^{1} (Bloomington stock 5138), *y, npr*^{3}*, FRT19A/FM7GG* and *usp*^{2}*, FRT19A/FM7a* and *w, usp*^{3}*, FRT19A; λ10, Tb/TM3Sb* and *w, usp*^{2}*, hs-Nmyc, FRT18A/FM7a* and *w, FRT18A; hs-Flp* (kindly provided by Schubiger), *−253/−39-lacZ* and *−348/−254Δ−112/−39-lacZ* (Cavaliere et al., 1997), *−444/−39-EcRE-M-lacZ*.

Clonal analyses

Clonal overexpression of *UAS-IR-EcR*, *UAS-IR-EcR-A*, *UAS-IR-EcR-B1* and *UAS-EcR-B2*^{F645A} was obtained using the Flp/Gal4 technique (Pignoni and Zipursky, 1997; Neufeld et al., 1998) by crossing the appropriate fly strains. Clonal overexpression of *UAS-IR-EcR* and of *UAS-IR-EcR-B1* was induced collecting respectively females of the genotype *hs-Flp/Act5C>CD2>Gal4; UAS-IR-EcR* and *hs-Flp/Act5C>CD2>Gal4; UAS-IR-EcR-B1/+*. Flies were heat shocked four times for 1 h at 37 °C and then were transferred to fresh vials with *y, w*^{67c23} males and incubated at 29 °C. Before dissection, the flies were transferred to fresh, yeasted food daily at 29 °C for 6 days. Clones overexpressing the *UAS-IR-EcR-A* transgene were obtained by heat shocking females of the genotype *Act5C>CD2>GAL4/hsFlp; UAS-IR-EcR-A/+* four times for 1 h at 37 °C. After the third heat shock, these females were transferred daily to fresh yeasted food at 29 °C for 10 days.

Clonal overexpression of *UAS-EcR-B2*^{F645A} was induced collecting females of the genotype *hs-Flp/Act5C>CD2>Gal4; UAS-EcR-B2*^{F645A}*/+*. Flies were heat shocked two times 1 h at 37 °C and then transferred to fresh vials with *y, w*^{67c23} males and incubated at 29 °C. Before dissection, the flies were transferred to fresh, yeasted food daily at 29 °C for 3 days.

Site-directed mitotic recombination was catalyzed by the heat shock-inducible Flp yeast recombinase at a FRT target element (Duffy et al., 1998). Females respectively of the genotype *w, usp*^{2}*, hs-Nmyc, FRT18A/w, Ubi-GFPnls, FRT18A; hs-Flp/−253/−39-lacZ* and *w, usp*^{2}*, hs-Nmyc, FRT18A/w, Ubi-GFPnls, FRT18A; hs-Flp/−444/−39-EcRE-M-lacZ* were heat-shocked four times and then transferred to fresh, yeasted food daily at 25 °C for 10 days.

usp^{2}, *usp*^{3} and *npr*^{3} clonal analyses were carried out using MARCM system (Lee and Luo, 1999). Females respectively of the genotype *usp*^{2}*, FRT19A/w, hs-Flp, tub-Gal80, FRT19A; UAS-mCD8-GFP/+; tub-Gal4/+* and *usp*^{2}*, FRT19A/w, hs-Flp, tub-Gal80, FRT19A; UAS-mCD8-GFP/−348/−254Δ−112/−39-lacZ; tub-Gal4/+* and *y, npr*^{3}*, FRT19A/w, hs-Flp, tub-Gal80, FRT19A; UAS-mCD8-GFP/+; tub-Gal4/+* were heat-shocked four times and then transferred daily to fresh, yeasted food at 25 °C for 10 days. Females *w, usp*^{3}*, FRT19A/w, hs-Flp, tub-Gal80, FRT19A; UAS-mCD8-GFP/+; tub-Gal4/+* were collected and heat-shocked three times. Before dissection the flies were transferred daily to fresh, yeasted food at 25 °C for 3 days.

Immunofluorescence microscopy

Fixation and antibody staining of hand-dissected ovaries were carried out as previously described (Andrenacci et al., 2001). Monoclonal anti-EcR 1:10 (AG10.2, DSHB), anti-EcR-A 1:10 (15G1A, DSHB), anti-EcR-B1 1:10 (AD4.4, DSHB), anti-CD2 1:250 (MCA154G, SEROTEC) antibodies were used and detected with FITC-conjugated anti-mouse secondary antibody (1:250, Invitrogen). Monoclonal anti-βgal 1:25 (401a, DSHB) was detected with Cy3-conjugated secondary antibody (1:200, Jackson). Anti-CVM32E (1:100), anti-VMP (1:50), anti-βgal (1:500, MP biomedicals) antibodies were detected with Cy3-conjugated anti-rabbit secondary antibody (1:100, Sigma). Stained egg chambers were mounted in Fluoromount-G (Electron Microscopy Sciences) for DAPI staining and subsequently were analyzed with conventional epifluorescence on a Nikon Eclipse 90i microscope and with TCS SL Leica confocal system.