Published in final edited form as:

Chromosome Res. 2013 July ; 21(4): 345–360. doi:10.1007/s10577-013-9360-1.

The ecdysone receptor (ScEcR-A) binds DNA puffs at the start of DNA amplification in *Sciara coprophila*

Gerald M. Liew,

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA

Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA

Michael S. Foulk, and

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA

Susan A. Gerbi

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA

Susan A. Gerbi: Susan_Gerbi@brown.edu

Abstract

The steroid hormone ecdysone induces DNA amplification and subsequent DNA puff formation in late fourth larval instar salivary gland polytene chromosomes of the fungus fly, *Sciara coprophila*. Previous in vitro studies on DNA puff II/9A in *Sciara* demonstrated that the ecdysone receptor (ScEcR-A) efficiently binds an ecdysone response element adjacent to the origin recognition complex binding site within the II/9A amplification origin, implying a role for ScEcR-A in amplification. Here, we extrapolate themolecular details from locus II/9A to the rest of the genome using immunofluorescence with a ScEcR-A-specific antibody. ScEcR-A binds all DNA puff sites just as amplification begins and persists throughout the processes of amplification, transcription, and puffing. Ecdysone injections into pre-amplification stage larvae prematurely induce both DNA amplification and ScEcR-A binding to DNA puff sites. These data are consistent with a direct role for ScEcR-A in DNA amplification.

Keywords

ecdysone; ecdysone receptor; *Sciara coprophila*; DNA amplification; immunofluorescence

© Springer Science+Business Media Dordrecht 2013

Correspondence to: Susan A. Gerbi, Susan_Gerbi@brown.edu.

This paper is dedicated to Natalia Gabrusewycz-Garcia whose beautiful work identified the DNA puffs in *Sciara* polytene chromosomes.

Electronic supplementary material The online version of this article (doi:10.1007/s10577-013-9360-1) contains supplementary material, which is available to authorized users.

Ethical Standards All experiments presented herein were performed in compliance with the current laws of the USA.

Conflict of Interest Gerald M. Liew, Michael S. Foulk, and Susan A. Gerbi declare that they have no conflict of interest. This article does not contain any studies with human or vertebrate animal subjects performed by any of the authors.

Introduction

Ecdysteroids play an important role in regulating the development and molting of insects. Particularly, in dipteran insects, the steroid hormone 20-hydroxyecdysone (henceforth referred to as ecdysone) induces the expression of genes required for metamorphosis during the last larval instar. Many types of insect tissue react to ecdysone at this developmental stage. Among the more prominent features to appear are puffs at specific sites in salivary gland polytene chromosomes. These RNA puffs form as a result of intense transcription (Pelling 1964) and are ecdysone inducible (Clever and Karlson 1960; Clever 1961; Clever 1965; Ashburner 1972). The binding of ecdysone to polytene chromosomal loci under its control was first shown by photoaffinity labeling experiments (Gronemeyer and Pongs 1980; Gronemeyer et al. 1981). According to the Ashburner model, ecdysone plays a dual role in puff formation in *Drosophila*, inducing early puffs and repressing late puffs. Activation of the early puffs produces transcription factors that trigger late puffs, leading to a cascade of RNA puffs across development (Ashburner et al. 1974; Kiss et al. 1976; Belyaeva et al. 1981; Thummel 1990). The early puffs are rapidly induced in a dosage-dependent manner and without a requirement for protein synthesis, suggesting that an ecdysone receptor is already present. The functional ecdysone receptor was later discovered to be a heterodimer of the ecdysone receptor (EcR) (Koelle et al. 1991) and ultraspiracle (USP) (Thomas et al. 1993; Yao et al. 1992, 1993). Recent genome-wide screening of EcR/USP binding sites in a *Drosophila* cell line has confirmed that many of its early targets encode factors that are involved in transcriptional regulation and puffing (Gauhar et al. 2009).

Besides RNA puffs, sciara flies also possess DNA puffs, so-called because they are sites of developmentally regulated DNA amplification in addition to transcription; DNA puffs have been previously identified by H3-thymidine uptake and quantified by microspectrophotometry and molecular methods (Breuer and Pavan 1955; Rudkin and Corlette 1957; Rasch 1970a; Glover et al. 1982; Lara et al. 1991; Gerbi et al. 1993; Wu et al. 1993; Stocker et al. 1996; de Almeida 1997). During the fourth and last instar of *Sciara coprophila* female larvae, the entire genome endoreduplicates up to 8,192 copies in each nucleus of the salivary glands without intervening mitoses (Rasch 1970b). The sister chromatids remain synapsed together in tight parallel array, forming giant polytene chromosomes that can be visualized under the microscope. During the last endocycle, specific origins of replication fire repeatedly, resulting in DNA amplification at those loci. Subsequent transcription of the “extra” DNA causes localized distension at those sites in the polytene chromosomes, resulting in gigantic DNA puffs (Poulson and Metz 1938; Gabrusewycz-Garcia 1964). Like RNA puffs in *Drosophila* (Ashburner et al. 1974), DNA puffs are regulated by ecdysone (Monesi et al. 2009) and can be prematurely induced by ecdysone injections into larvae, as demonstrated on both the cytological level (Crouse 1968; Stocker and Pavan 1974; Amabis and Amabis 1984a) and the molecular level (Foulk et al. 2006). Moreover, DNA puffing does not occur if the flow of ecdysone from the prothoracic gland is physically restricted by a ligature posterior to the brain (Amabis and Amabis 1984b).

Eighteen DNA puffs arise at specific loci in *Sciara* salivary gland polytene chromosomes near the end of the fourth larval instar (Table 1) (Gabrusewycz-Garcia 1964, 1971). The largest and earliest forming puff is located on chromosome II at position 9A (II/9A), and we have characterized the molecular structure of this puff site and its response to ecdysone. The II/9A locus contains two genes (II/9-1 and II/9-2) which share 85 % sequence similarity and encode secretory proteins with α -helical coiled coil regions that are presumably secreted to form the pupal case (DiBartolomeis and Gerbi 1989). Previous two- and three-dimensional gel mapping studies defined a 1-kb origin (ORI) where replication initiates during DNA amplification (Liang et al. 1993; Liang and Gerbi 1994). A DNase I hypersensitive site is

located about 600 bp upstream of the 1-kb ORI (Urnov et al. 2002) and may serve as an upstream border for the initiation zone of DNA synthesis at all stages of development (Lunyak et al. 2002). A binding site for the origin recognition complex (ORC) is present within the 5' end of the 1-kb ORI and is adjacent to the start site for bidirectional DNA replication, localized at the nucleotide level by replication initiation point mapping (Bielinsky et al. 2001). Additionally, a putative ecdysone response element (EcRE) is situated adjacent to the ORC binding site within the 1-kb ORI (Foult et al. 2006). This ORI EcRE is capable of being bound by the *Sciara* EcR/USP complex, as shown by gel shifts, suggesting that ecdysone might act through this EcRE to regulate the amplification process (Foult et al. 2006). EcREs are also present in the promoters of genes II/9-1 and II/9-2 (DiBartolomeis and Gerbi 1989; Bienz-Tadmor et al. 1991). Isoform A of the ecdysone receptor (ScEcR-A) predominates in the salivary glands of fourth instar *Sciara* larvae (Foult et al. 2006, 2013). At the earliest forming DNA puffs, II/9A and II/2B, DNA amplification initiates just before morphological puffing becomes evident (stage 10×5), and replication continues through the onset of puff formation (stage 12×6) and plateaus when a burst of transcription results in maximal puff expansion (stage 14×7) (Wu et al. 1993).

In order to extrapolate the molecular details elucidated from the II/9A locus to other amplified loci, we have conducted immunofluorescence experiments to visualize the binding of ScEcR-A in salivary gland polytene chromosomes during the late fourth larval instar. Moreover, immunofluorescence on polytene chromosomes provides a way to acquire global, genome-wide information about sites bound by the hormone receptor in the absence of the complete *Sciara* genome sequence, which is necessary for ChIP-seq. We reasoned that the appearance of ScEcR-A at DNA puff sites at the start of amplification, but before transcription or puffing, would further support its direct involvement in the amplification process. We find that ScEcR-A localizes to DNA puff sites at stage 10×5, just as amplification is starting. These signals persist throughout the processes of amplification, transcription, and puffing. ScEcR-A also binds to RNA puff sites and to other sites in the genome, consistent with its role as a transcription factor and the major regulator of insect development. Finally, ecdysone injections into pre-amplification stage larvae prematurely induce the natural progression of ScEcR-A signals across the genome, supporting our hypothesis that ScEcR-A might be involved in DNA amplification.

Materials and methods

Fly stocks

S. (Bradysia) coprophila stock 7298 (wild type with Wavy wing marker linked to the X' inversion chromosome) and 7298 derivative stocks ("Holo") were raised in the laboratory at 21 °C. Only female fourth instar larvae from these stocks were used for immunofluorescence studies because they undergo an additional endoreduplication cycle compared to males (Rasch 1970b), resulting in larger polytene chromosomes. Well-fed larvae grown in medium density conditions yielded chromosome squashes of significantly better quality. The expansion of puff morphology coincides with development of the eye anlage from mid to late fourth larval instar, allowing larvae to be staged by counting their eyespot granules under a 16× objective. Each developmental stage is specified by the number of eyespot granules in the longest row multiplied by the number of rows minus one (Gabrusewycz-Garcia 1964; Wu et al. 1993). At the earliest DNA puffs II/2B and II/9A, amplification initiates at eyespot stage 10×5 and continues through 12 × 6, while maximal puffing resulting from a burst of transcription occurs at the 14×7 stage (Gerbi et al. 1993; Wu et al. 1993; Foult et al. 2006).

Antibodies

ScEcR-A is isoform A of EcR, and it predominates in the salivary glands of fourth larval instar *S. coprophila* (Foulk et al. 2013). A polyclonal antibody to this isoform was produced against the unique ScEcR-A NH₂-terminus as described (Foulk et al. 2013). Alexa Fluor 488 dye-conjugated goat anti-rabbit IgG was obtained from Molecular Probes (Invitrogen, Eugene, OR, USA). Anti-actin antibody, clone C4, was obtained from EMD Millipore (MAB 1501; Billerica, MA, USA).

Immunoblotting

Approximately 30 pairs of salivary glands from each developmental stage were dissected in Robert's CR buffer (87 mM NaCl, 3.2 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 10 mM Tris-HCl, pH 7.3) (Robert 1971) for immunoblotting. Equal loading of overall protein in each lane was facilitated with the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The ColorPlus Prestained Protein Ladder (New England Biolabs, Ipswich, MA, USA) was used as molecular weight marker. Crude fractionation of salivary glands was carried out as follows: salivary gland cell suspensions were made by homogenizing whole salivary glands with an Eppendorf tube pestle in Robert's CR buffer and pelleted by centrifugation. Cells were swollen in 50 µl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1× protease inhibitor cocktail [Roche Diagnostics GmbH, Mannheim, Germany]) for 50 min on ice. Cells were lysed by adding 1.5 µl Igepal CA-630 (Nonidet P-40; Sigma, St. Louis, MO, USA) and vortexing. Nuclei were pelleted by centrifugation for 30 s at 10,000 rpm and the supernatant was saved as the cytoplasmic fraction.

Ecdysone injections

Ecdysone injections were performed as described by Foulk et al. (2006). Pre-amplification stage larvae (early eyespot or 8×4 stages) were micro-injected with 320 pg of 20-hydroxyecdysone (Sigma) in 50 % ethanol, together with 25 mg/ml blue dextran (Sigma) as a tracer. Control larvae were microinjected with 50 % ethanol containing blue dextran as a tracer. The total volume injected into each larva was 32 nl. After injection, larvae were kept in small Petri dishes on 2.2 % Bacto agar (Becton Dickinson Company, Sparks, MD, USA) at 21 °C for 12–18 h.

Preparation of chromosome squashes and immunofluorescent detection of ScEcR-A

Polytene chromosome squashes and immunostaining were performed as described by Zink et al. (1991) with modifications. Briefly, the anterior portions of salivary glands were excised from staged larvae in dissecting buffer (PBS, pH 7.4, 0.1 % Triton X-100) [PBS, 137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄, pH 7.4] and immediately fixed for 25 s in freshly prepared solution I (3.7 % formaldehyde, 1 % Triton X-100, PBS, pH 7.4). Glands were then transferred to a 35-µl drop of solution II (3.7 % formaldehyde, 50 % acetic acid) on a siliconized coverslip for 70 s. The 37 % formaldehyde stock solution used for solutions I and II was made fresh before each experiment by boiling paraformaldehyde (Sigma) in 15 mM KOH until dissolved. After picking up the coverslip with a poly-L-lysine-coated slide [slide coating: clean slides were soaked for 5 min in 0.1 % poly-L-lysine (Sigma) and oven dried at 80 °C for 1 h], the entire preparation was flipped over. Chromosomes were spread by firmly tapping the coverslip around the glands with a pencil eraser. The chromosomes were then flattened by compression of the slide between several sheets of blotting paper with thumb pressure. After freezing the preparation in liquid nitrogen, the coverslip was pried off with a razor blade and the exposed chromosome squash was washed in PBS (2×15 min, agitating). Slides were then blocked (PBS, pH 7.4, 3 %

nonfat dried milk, 0.3 % Triton X-100) for 1 h at room temperature or overnight at 4 °C and washed (2×10 min, agitating) with PBT (PBS, pH 7.4, 0.1 % BSA, 0.2 % Tween-20).

Chromosome preparations were reacted sequentially with 40 μ l anti-ScEcR-A (1:2,500 in PBT) and then Alexa Fluor 488 dye-conjugated goat anti-rabbit IgG (1:1,000 in PBT) for 1 h each. Each immunostaining step was followed by a PBT wash (2×10 min, agitating). Slides were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; 200 ng/ml in H₂O) for 10 min. All incubation steps were carried out at room temperature in a darkened humid chamber. After a brief rinse in PBT, slides were mounted with ProLong[®] Gold (Invitrogen, Carlsbad, CA, USA) and stored in the dark at 4 °C. Reacted preparations were examined under a Carl Zeiss Axiovert 200M microscope and photographed with the 40 $\times$ objective (dry). AxioVision software was used to optimize exposure times automatically according to the immunostaining intensity of each chromosome preparation. Captured images were then exported into Adobe Photoshop[®] for resizing and cropping.

Results

ScEcR-A is the predominant isoform of ecdysone receptor found in *Sciara* larval salivary glands (Foulk et al. 2013). A polyclonal antibody was produced against the unique NH₂-terminus of the ScEcR-A protein. This antibody detected the 68-kDa band of ScEcR-A in *Sciara* salivary gland extracts as determined by Western blot (Fig. 1a) and did not cross-react with the other *Sciara* ecdysone receptor isoform, ScEcR-B, that is 61 kDa. The ScEcR-A antibody also bound a cross-reacting band of about 49 kDa (asterisk; Fig. 1a) and high molecular weight, weakly cross-reacting bands (detected on long exposures; Online Resource 1) at every developmental stage examined by Western blot. However, crude fractionation of the salivary glands into cytoplasmic and nuclear fractions showed that ScEcR-A is enriched in the nuclear fraction while the 49-kDa cross-reacting band is depleted (Fig. 1b). Moreover, since no immunofluorescent signal was observed on salivary gland polytene chromosomes at pre-eyespot (PES) or early eyespot (EES) stages (Figs. 2, 3, 4, and 5) despite the presence of cross-reacting bands on the Western blot at these stages (Fig. 1a), we conclude that the cross-reacting bands do not give rise to staining on the chromosomes. Therefore, the signals observed on the polytene chromosomes are specific to bound ScEcR-A. Control experiments further confirmed that the chromosomal signals were specifically due to ScEcR-A. Incubation of chromosome squashes prepared from larvae at 14 $\times$ 7 stage with pre-immune serum and secondary antibody resulted in weak, general fluorescence, but no distinct labeling of chromosomes (Online Resource 2a–c). Reaction of 14 $\times$ 7 stage preparations with the secondary antibody alone gave a similar result of no signal (Online Resource 2d–f).

The ScEcR-A polyclonal antibody was used to perform immunofluorescence experiments to detect the binding of ScEcR-A to polytene chromosomes II, III, IV, and X (Figs. 2, 3, 4, and 5, respectively) during late larval development in *S. coprophila*. We found that the level of chromosome-associated ScEcR-A increased in salivary gland chromosomes during the fourth larval instar, mirroring the increase in ScEcR-A levels in the salivary glands themselves. Pre-amplification stage (8 $\times$ 4) larvae exhibited few ScEcR-A-specific fluorescent bands per chromosome. Increased ScEcR-A staining across the genome occurred in 10 $\times$ 5 and 12 $\times$ 6 stage larvae, with maximal intensity of immunostaining seen in 14 $\times$ 7 stage larvae (see Figs. 2, 3, 4, and 5).

ScEcR-A associates with DNA puffs

First, we analyzed ScEcR-A immunostaining at the DNA puff loci in *Sciara* salivary glands from various developmental stages. We have followed the previous nomenclature for both DNA puffs and RNA puffs (Gabrusewycz-Garcia 1964; Gabrusewycz-Garcia and Kleinfeld

1966), in which DNA puffs were identified by autoradiography following H^3 -thymidine incorporation and RNA puffs by H^3 -uridine incorporation. The chromosomes of early eyespot stage larvae (well before DNA puff amplification) usually showed no ScEcR-A immunostaining, except for a single pale band at future DNA puff site IV/19A if any signal was detected at all (Online Resource 2g–i). This signal was also consistently seen as a bright band in preparations from 8×4 stage larvae (Fig. 4c). Other consistent pale signals at the 8×4 stage arose at DNA puffs III/15B, IV/8C, and IV/10B (Figs. 3c and 4c). Long exposures of ScEcR-A Western blots (Online Resource 1) revealed a very weak ScEcR-A signal at the PES, EES, and 8×4 stages. The average exposure time required for detection of signal at the 8×4 stage was approximately 4.5 times longer than that for the other stages (Online Resource 3), reflecting the very low level of ScEcR-A during this stage of development (Online Resource 1).

Importantly, immunostaining of all major and minor DNA puff loci generally occurred at the 10×5 stage. ScEcR-A staining during this stage usually coincided with preliminary puff formation at two of the largest and earliest forming DNA puffs, II/2B and II/9A (Fig. 2f). Loci of DNA puff sites that gave strong signals during the 10×5 stage were II/2B, II/9A, and II/11A (Fig. 2f); III/2B, III/11A, and III/15B (Fig. 3f); and IV/8C, IV/10B, IV/12A, and IV/19A (Fig. 4f). Signals of pale to modest strength at stage 10×5 were seen at other DNA puff sites including II/6A, II/13A, and II/14B (Fig. 2f); III/10A (Fig. 3f); IV/5C and IV/15B (Fig. 4f); and X/7A and X/11B (Fig. 5f). Most DNA puffs are present in the anterior and posterior portions of the salivary glands, but a few are found only in the anterior or posterior parts (Table 1). Interestingly, ScEcR-A was consistently detected at loci II/11A, II/14B, and IV/15B in anterior portions of salivary glands from stage 10×5 (Figs. 2f and 4f), despite the fact that these loci only puff morphologically in chromosomes from the posterior segment of the salivary gland (Gabrusewycz-Garcia 1964, 1971).

The signal strength at most DNA puff sites tended to increase from stages 10×5 through 14×7, paralleling the expansion of puff morphology (Figs. 2, 3, 4, and 5). As seen in separate channels, certain DNA puffs showed both increased levels of DAPI and anti-ScEcR-A staining, e.g., II/2B, II/6A, II/9A, III/2B, and III/11A (Figs. 2l and 3l). Yet, other DNA puffs exhibited diffuse interiors under DAPI staining, allowing ScEcR-A signals to be seen easily in the merged image, e.g., III/15B (Fig. 3l). Several DNA puffs, however, showed little or no increase in ScEcR-A signal strength over this time frame. These were mostly found on chromosome IV, e.g., IV/5C and IV/12A (Fig. 4l). Overall, the developmental profile of ScEcR-A immunofluorescence was entirely reproducible (Online Resources 4, 5, and 6).

ScEcR-A associates with RNA puffs

ScEcR-A was found to bind most RNA puff sites in *Sciara* during the fourth larval instar. Unlike DNA puffs, RNA puffs do not arise in synchrony during development (Gabrusewycz-Garcia 1964). At the 8×4 stage, ScEcR-A signals could be seen at III/9B (Fig. 3c), IV/9C, and IV/10A (Fig. 4c), suggesting the involvement of ScEcR-A in transcription at those loci.

Many RNA puff sites exhibited moderate to strong signals from stages 10×5 through 14×7. The positions of these signals on different chromosomes are as follows: 1B, 10B/C, 12A, 12B, and 13C on chromosome II (Fig. 2); 4A, 5C, 7A, 9B, and 12C on chromosome III (Fig. 3); and 4C, 5B, 6B, 7B, 9C, 10A, 10C, and 19C on chromosome IV (Fig. 4). Notably, RNA puff III/9B consistently exhibited a signal that was intense enough to rival even the brightest DNA puffs (Fig. 3). In some preparations, ScEcR-A labeled this puff slightly off-center, similar to experimental results seen by *in situ* hybridization with a cDNA probe against this

locus (Wu et al. 1993). Analysis of the RNA puffs on the X chromosome was omitted because the triple repeat regions make analysis of ScEcR-A binding difficult.

ScEcR-A binds to other regions of the genome

Examination of immunostained preparations showed that ScEcR-A bound to some regions of the genome that did not correspond to previously described DNA or RNA puffs (Figs. 2, 3, 4, and 5). These “orphan” signals formed a consistent and reproducible banding pattern, providing a convenient way of identifying and mapping each chromosome according to the charts made by Gabrusewycz-Garcia (1964). Many of these orphan signals were observed in the polytene chromosomes, reflecting the role of the ecdysone receptor as the master regulator of development. Two of the brightest of these non-puff signals were found at II/4A (Fig. 2) and III/13C (Fig. 3) and were observed across development. The signal at II/4A was seen to appear as early as the 8×4 stage (Fig. 2c). Another weak orphan signal was observed at the 8×4 stage at IV/14C (Fig. 4c). A summary of all prominent observed ScEcR-A signals is presented in Table 1 and depicted graphically in Fig. 6.

Ecdysone injections into pre-amplification stage larvae prematurely induce the natural progression of ScEcR-A signals across the genome

Ecdysone injections into the hemolymph of individual pre-amplification stage *Sciara* larvae (early eyespot or 8×4 stages) have been previously shown to induce premature DNA amplification (Foulk et al. 2006) and puffing (Crouse 1968). In particular, robust amplification was observed at 18 h after ecdysone injection, suggesting the possible involvement of the ecdysone receptor in amplification (Foulk et al. 2006). Could ecdysone injection into pre-amplification stage larvae prematurely induce ScEcR-A signals similar to those observed in 10×5 stage larvae undergoing amplification? To answer this question, we injected 320 pg of ecdysone in 50 % ethanol (in a total volume of 32 nl) into individual pre-amplification stage larvae and performed chromosome squashes and ScEcR-A immunostaining at 12, 15, 18, and 24 h post-injection. Control larvae were mock injected with 32 nl of 50 % ethanol.

We found that ecdysone injections into pre-amplification stage larvae accelerated the normal transition from the early eyespot/8×4 stage to the edge eye stage. Larvae usually take 4 days to transit from the 8×4 stage to the edge eye stage. This transition occurred over a time span of 24 h when 8×4 stage larvae were injected with ecdysone demonstrating that ScEcR-A signals could be prematurely induced by ecdysone injection as well. At 12 h post-injection, a few ScEcR-A signals were observed across the genome (Fig. 7a). Loci displaying signals at this time included II/4A, II/12B, III/15B, and IV/19A (Fig. 7a), similar to the signals seen at the 8×4 stage (Figs. 2c, 3c, and 4c). At 15 h post-injection, ScEcR-A signals were observed mirroring the overall trend of ScEcR-A signals seen in larvae from stages 10×5 through 14×7 (Fig. 7b and data not shown). Notably, the strength and intensity of ScEcR-A signals at DNA puff sites during this time were comparable to the natural progression of signals in 10×5 stage larvae. At 18 h post-injection, a weaker, more general ScEcR-A immunofluorescence was observed over the entire genome (Fig. 7c), similar to the decrease in overall ScEcR-A signal in edge eye stage larvae observed by immunoblotting (Foulk et al. 2013). The chromosomes of mock-injected larvae showed no ScEcR-A staining up to 18 h post-injection (Online Resource 2j–l). Finally, because ecdysone prematurely induces pupation and histolysis of salivary glands, we found that the polytene chromosomes at 24 h post-injection were not useful for ScEcR-A immunofluorescence (results not shown).

Discussion

Ecdysone induces amplification and morphological puffing in salivary gland polytene chromosomes of *S. coprophila* during late fourth larval instar (Crouse 1968; Foulk et al. 2006). However, the exact mechanism by which the cellular controls normally preventing re-replication are subverted during this process remains unclear. It has been shown previously that ecdysone induces amplification at DNA puff II/9A, and the identification of a putative EcRE adjacent to the ORC-binding site within the 1-kb ORI of II/9A further implied a direct role for the ecdysone receptor in the amplification response (Foulk et al. 2006). Isoform A of the ecdysone receptor (ScEcR-A) predominates in the salivary glands of fourth instar *Sciara* larvae (Foulk et al. 2013). In this study, we asked if the ecdysone receptor binds to the other DNA puffs at the onset of DNA amplification, consistent with the molecular details elucidated from the II/9A locus suggesting a direct role for ScEcR-A as the amplification factor. To answer that question at the genome level in the absence of a complete *Sciara* genome sequence, we performed immunofluorescence experiments to visualize the binding of ScEcR-A on late fourth larval instar salivary gland polytene chromosomes. We have compiled here (Table 1) a list of DNA puffs and RNA puffs that have been described previously (Gabrusewycz-Garcia 1964, 1971) as well as a compilation of the sites of ScEcR-A binding discovered here in *S. coprophila* polytene chromosomes (Fig. 6). At the early eyespot stage, a weak ScEcR-A band was usually observed at IV/19A if any signal was detected at all. At stage 8×4, weak ScEcR-A signals appeared at select sites across the genome. Some of these sites correspond to DNA puff loci (III/15B, IV/8C, IV/10B, IV/19A) and RNA puff loci (III/9B, IV/9C, IV/10A), while other sites (II/4A, IV/14C) are neither DNA nor RNA puff loci but have a high affinity for ScEcR-A at stage 8×4. Importantly, strong ScEcR-A signals localizing to all DNA puffs and most RNA puffs occurred at stage 10×5 (when DNA amplification initiates; Wu et al. 1993), and persisted through to stage 14×7. ScEcR-A also bound to some other sites across the genome, giving rise to orphan signals. Furthermore, ecdysone injections into pre-amplification stage larvae prematurely induced the natural progression of all the ScEcR-A signals reported here.

The persistence of ScEcR-A signals throughout amplification, transcription, and puffing at specific DNA puff sites suggests the involvement of ScEcR-A in these three processes. Although most DNA puff loci tended to show increased ScEcR-A signal strength from stage 10×5 through stage 14×7, the intensity of ScEcR-A signal at individual puffs did not always correlate with the extent and timing of morphological puffing. Recently, it was shown that the conformation and activity of another steroid hormone receptor—the glucocorticoid receptor—is regulated by variations in the DNA sequences it binds to (Meijsing et al. 2009). Perhaps a similar scenario exists in *Sciara* as well, where the role of ScEcR-A in directing the ecdysone response is influenced by the unique sequence characteristics of each puff site. In particular, the different strengths of ScEcR-A binding at different sites in the genome could result from two possibilities: (a) differences in the EcRE sequences at different puff sites that confer distinct affinities for ScEcR-A or (b) differences in the number of EcREs at each ScEcR-A binding site, with more EcREs allowing more ScEcR-A to be bound.

Recent genome-wide mapping of binding sites for the ecdysone receptor in *Drosophila* Kc cells identified 502 major binding regions and showed that addition of ecdysone induced or repressed transcription of 113 or 59 genes, respectively, as an early response (Gauhar et al. 2009) in good agreement with another recent study (Gonsalves et al. 2011). In *Drosophila* salivary glands, genomic analysis revealed 87 genes induced and 9 repressed by ecdysone with little overlap with the ecdysone-regulated genes in Kc cells (Gonsalves et al. 2011). The number of ecdysone-regulated genes in *Drosophila* salivary glands analyzed by another group was larger, but reflects a higher ecdysone concentration and a longer incubation time (Beckstead et al. 2005). By comparison, we observed approximately 180 ScEcR-A signal

bands by immunofluorescence in *Sciara*, which is in good agreement with the ~100 ecdysone-regulated loci identified by others in *Drosophila*. Cytological resolution does not allow us to directly correlate the observed ScEcR-A signal bands to the number of EcRE sites, as there could be several EcRE sites for a given EcR immunofluorescent band. The relationship of the observed ScEcR-A immunofluorescence signals to the actual binding sites must await the completion of the *Sciara* genome sequence, which is not yet available.

The identification of numerous ScEcR-A orphan signals across the *Sciara* genome, such as at II/4A and III/13C, raises a question as to their presence. These signals likely represent heretofore unidentified genes that are regulated by ScEcR-A, reflecting the role of ecdysone and the ecdysone receptor as the master regulator of development. On the other hand, Gauhar et al. (2009) discovered that only 42 % of the ecdysone receptor bound sites coincided with ecdysone-regulated genes in *Drosophila* Kc cells, suggesting that tissue-specific factors are needed in addition to EcR to regulate transcription. Conceivably, some of the orphan signals that we observed in *Sciara*, as well as ScEcR-A signals for puffs active only in the posterior portion of the salivary gland, could correspond to ecdysone-regulated genes that are potentially expressed in other *Sciara* cell types, but not in the anterior portion of the salivary glands where the tissue-specific cofactor would be absent. Although these sites are bound by ScEcR-A, they may not be expressed due to the lack of the necessary cofactors.

Is the ecdysone receptor involved in amplification?

ScEcR-A bound all known DNA puff sites at eyespot stage 10×5, when amplification but not transcription was occurring at II/9A. In addition, ecdysone injections into pre-amplification stage larvae prematurely induced the normal course of ScEcR-A signals at all DNA puff sites, including II/9A. These results are consistent with a direct role for the ecdysone receptor in regulating DNA amplification. Moreover, the timing and intensity of ScEcR-A binding to II/9A are comparable to that of II/2B, a DNA puff that exhibits an amplification and transcription profile similar to II/9A (Wu et al. 1993). This leads us to believe that amplification at other loci might be activated in a similar way to II/9A, despite the fact that the amplification and transcription developmental profiles of these other loci have yet to be characterized.

Two previous attempts have been made to visualize the ecdysone receptor at the sites of DNA puffs during the start of amplification in different species of sciarid flies. In the first study, Stocker et al. (1997) used a *Chironomus* EcR-specific antibody to detect the ecdysone receptor in *Trichosia pubescens*, while in the second study Candido-Silva et al. (2008) developed a *Bradysia hygida* EcR-specific antibody and used it to study association of the ecdysone receptor within the same organism. Both studies revealed binding of the ecdysone receptor to DNA puffs during transcription, but no significant signal was observed at loci initiating DNA amplification prior to transcription. In contrast, we have demonstrated the binding of the ecdysone receptor to DNA puff sites at the beginning of amplification (stage 10×5) until transcription (stage 14×7) with significant signals already seen over the DNA puffs at stage 10×5 when amplification starts. Our results could be due to better antibody specificity, better resolution of developmental stages, or differences in amplification biology between *Sciara* and the two species of fly previously studied.

Our finding that ScEcR-A appears at DNA puffs when DNA amplification is initiating is consistent with a direct role for ScEcR-A in the mechanism for gene amplification. Ecdysone could first upregulate ScEcR-A levels, following which ScEcR-A would bind to the EcRE adjacent to the replication ORI (Foult et al. 2006) where it may induce DNA amplification by interacting with components of the replication machinery within the ORI. In fact, a putative EcR binding site is known to be located adjacent to the ORI at the II/9A

locus (Foult et al. 2006). The ecdysone receptor was originally identified in *Drosophila* as a transcription factor, directing the expression of ecdysone-responsive genes (Koelle et al. 1991). Thus, binding of ScEcR-A to the II/9A 1-kb ORI EcRE and other DNA puff ORIs during amplification would suggest a new role, besides transcription, for the ecdysone receptor in regulating DNA replication. The ecdysone receptor has also been implicated in DNA amplification in *Drosophila* follicle cells where genetic ablation of DmEcR-B1 prevents DNA amplification (Hackney et al. 2007). Whether this is a direct or indirect effect remains unknown, despite the intriguing observation that amplification control element 3 in *Drosophila* contains a match to the consensus EcRE (Hackney et al. 2007).

Other untested mechanisms also remain possible whereby EcR could induce DNA amplification. Since the transcription factor function of EcR is to recruit RNA polymerase II (Pol II) for transcription, the question arises whether EcR recruitment of Pol II would also induce DNA amplification. In *Sciara*, DNA amplification precedes transcription at DNA puffs II/9A and II/2B (Wu et al. 1993) that are two of the earliest DNA puffs. Moreover, inhibition of Pol II by a low concentration of actinomycin D does not prevent ecdysone induction of DNA amplification of locus II/9A in explanted salivary glands from *Sciara* (Foult et al. 2006). Therefore, transcription is not observed at the onset of DNA puff amplification, nor is it required at *Sciara* DNA puff II/9A. Although the transcriptional timing is not yet known for the other DNA puffs, evidence from *Drosophila* follicle cells also suggests that transcription is not a universal prerequisite for DNA amplification. Although α -amanitin blocks stage 13 amplification at DAFC-62D, it does not block the earlier burst of DNA amplification at stage 10 (Xie and Orr-Weaver 2008). Moreover, even though *Drosophila* DAFC-34B also has two periods of DNA amplification, neither of them is sensitive to α -amanitin (Kim and Orr-Weaver 2011).

Even though transcription follows DNA amplification in *Sciara* DNA puffs II/2B and II/9A, and transcription is usually not required for DNA amplification in *Drosophila* follicle cells as discussed above, the possibility remains that EcR could recruit Pol II that is transcriptionally inactive during the onset of DNA amplification but necessary nonetheless. The occurrence of stalled Pol II is common in the genomes of various metazoans (Lis 2007; Levine 2011; Adelman and Lis 2012). In many cases, nuclear run-on experiments have shown that the polymerase is simply paused (often proximal to the promoter) and can resume transcription after Sarkosyl treatment to remove pausing factors (Adelman and Lis 2012). Paused Pol II helps to maintain chromatin in an open state (Adelman and Lis 2012). Not only transcription factors, but also DNA replication factors needed for DNA amplification might be more readily recruited to such open chromatin. Moreover, replication origins are often found near promoters for transcription. Specifically, in the case of *Sciara* DNA puff II/9A, the DNA amplification origin is located slightly upstream of the promoter for gene II/9-1 (Liang et al. 1993; Lunyak et al. 2002). Similarly, the six amplification origins in *Drosophila* follicle cells coincide with Pol II occupancy (Kim et al. 2011). In *Sciara*, chromatin immunoprecipitation revealed that Pol II is located at the gene II/9-1 promoter at stage 10 $\times$ 5 when DNA amplification begins, even though transcription from this locus is not observed until after the completion of DNA amplification (Lunyak et al. 2002).

Chromatin could be opened up by Pol II, but it also could be opened up simply by EcR itself. A mark of open chromatin, hyperacetylated histone H4 (especially H4K8), is enriched at *Drosophila* follicle cell amplicons and correlates with DNA amplification levels (Aggarwal and Calvi 2004; Hartl et al. 2007; Kim et al. 2011). However, only two of the six *Drosophila* follicle cell amplicons show significant enrichment of H4 acetylation with the region bound by the ORC, suggesting that H4 acetylation is not sufficient or necessary for ORC localization, which is considered a prerequisite for origin firing (Kim et al. 2011). Finally, no histone acetyltransferases have been reported to date to bind to EcR. Taken

together, these points suggest that EcR probably does not induce DNA re-replication, resulting in DNA amplification, by H4 acetylation.

The data reported here demonstrate that EcR-A binds to loci in the *Sciara* genome at the time when DNA amplification is initiating, thus mediating the known induction of *Sciara* DNA puff amplification by ecdysone (Foulk et al. 2006). The existence of an EcRE that can bind ScEcR-A at the *Sciara* II/9A amplification origin is consistent with the hypothesis that ScEcR-A might interact directly with the replication machinery. As discussed above, it is also possible that ScEcR-A which is bound to DNA puff loci during the initiation of DNA amplification might promote DNA amplification through its potential recruitment of a paused Pol II. The data presented here will serve as a foundation for future studies to unravel the precise mechanism through which ecdysone induces DNA amplification. In any case, evidence described here suggests a direct role for ScEcR-A rather than simply an indirect role via its induction of a transcriptional cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

G.M.L. was supported by a fellowship from the Agency for Science, Technology and Research (A*STAR), Singapore.

Abbreviations

DAPI	4', 6-Diamidino-2-phenylindole
EcR	Ecdysone receptor
EcRE	Ecdysone response element
ORC	Origin recognition complex
ORI	Origin of replication
Pol II	RNA polymerase II
USP	Ultraspiracle

References

- Adelman K, Lis JT. Promoter-proximal pausing of RNA polymerase II: emerging roles in metazoans. *Nat Rev Genet.* 2012; 13:720–731. [PubMed: 22986266]
- Aggarwal BD, Calvi BR. Chromatin regulates origin activity in *Drosophila* follicle cells. *Nature.* 2004; 430:372–376. [PubMed: 15254542]
- Amabis DC, Amabis JM. Effects of ecdysterone in polytene chromosomes of *Trichosia pubescens*. *Dev Biol.* 1984a; 102:1–9. [PubMed: 6698299]
- Amabis DC, Amabis JM. Hormonal control of gene amplification and transcription in the salivary gland chromosomes of *Trichosia pubescens*. *Dev Biol.* 1984b; 102:10–20. [PubMed: 6698300]
- Ashburner M. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila* VI. Induction by ecdysone in salivary glands of *Drosophila melanogaster* cultured in vitro. *Chromosoma.* 1972; 38:225–281.
- Ashburner M, Chihara C, Meltzer P, Richards G. Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harb Symp Quant Biol.* 1974; 38:655–662. [PubMed: 4208797]
- Beckstead RB, Lam G, Thummel CS. The genomic response to 20-hydroxyecdysone at the onset of *Drosophila* metamorphosis. *Genome Biol.* 2005; 6:R99. [PubMed: 16356271]

- Belyaeva ES, Vlassova IE, Biyasheva ZM, Kalpakov VT, Richards G, Zhimulev IF. Cytogenetics analysis of the 2B3-4-2B11 region of the X chromosome of *Drosophila melanogaster*. *Chromosoma*. 1981; 84:207–219. [PubMed: 6799261]
- Bielinsky AK, Blitzblau H, Beall EL, Ezrokhi M, Smith HS, Botchan MR, Gerbi SA. Origin recognition complex binding to a metazoan replication origin. *Curr Biol*. 2001; 11:1427–1431. [PubMed: 11566101]
- Bienz-Tadmor B, Smith HS, Gerbi SA. The promoter of DNA puff II/9-1 of *Sciara coprophila* is inducible by ecdysone in late prepupal salivary glands of *Drosophila melanogaster*. *Cell Regul*. 1991; 2:875–888. [PubMed: 1809395]
- Breuer ME, Pavan C. Behavior of polytene chromosomes of *Rhynchosciara angelae* at different stages of larval development. *Chromosoma*. 1955; 7:371–386.
- Candido-Silva JA, de Carvalho DP, Coelho GR, de Almeida JC. Indirect immune detection of ecdysone receptor (EcR) during the formation of DNA puffs in *Bradysia hygida* (Diptera, Sciaridae). *Chromosome Res*. 2008; 16:609–622. [PubMed: 18483872]
- Clever U. Gene activity in the giant chromosomes of *Chironomus tentans* and its relation to development I. Gene activation by ecdysone. *Chromosoma*. 1961; 12:607–675. [PubMed: 13879936]
- Clever U. Puffing changes in incubated and in ecdysone treated *Chironomus tentans* salivary glands. *Chromosoma*. 1965; 17:309–322. [PubMed: 5885997]
- Clever U, Karlson P. Induction of puff changes in the salivary gland chromosomes of *Chironomus tentans* by ecdysone. *Exp Cell Res*. 1960; 20:623–626. [PubMed: 13694081]
- Crouse HV. The role of ecdysone on DNA-puff formation and DNA synthesis in the polytene chromosomes in *Sciara coprophila*. *Proc Natl Acad Sci USA*. 1968; 61:971–978. [PubMed: 5246561]
- de Almeida JC. A 28-fold increase in secretory protein synthesis is associated with DNA puff activity in the salivary gland of *Bradysia hygida* (Diptera, Sciaridae). *Braz J Med Biol Res*. 1997; 30:605–614. [PubMed: 9283627]
- DiBartolomeis SM, Gerbi SA. Molecular characterization of DNA puff II/9A genes in *Sciara coprophila*. *J Mol Biol*. 1989; 210:531–540. [PubMed: 2614832]
- Foulk MS, Liang C, Wu N, Blitzblau HG, Smith H, Alam D, Batra M, Gerbi SA. Ecdysone induces transcription and amplification in *Sciara coprophila* DNA puff II/9A. *Dev Biol*. 2006; 299:151–163. [PubMed: 16938289]
- Foulk MS, Waggener JM, Johnson JM, Yamamoto Y, Liew GM, Urnov FD, Young Y, Lee G, Smith HS, Gerbi SA. Isolation and characterization of the ecdysone receptor and the heterodimeric partner ultraspiracle through development in *Sciara coprophila*. *Chromosoma*. 2013; 122:103–119. [PubMed: 23321980]
- Gabrusewycz-Garcia N. Cytological and autoradiographic studies in *Sciara coprophila* salivary gland chromosomes. *Chromosoma*. 1964; 15:312–344. [PubMed: 14198770]
- Gabrusewycz-Garcia N. Studies of polytene chromosomes of sciarids. I. The salivary gland chromosomes of *Sciara (Lycoriella) pauciseta* (II), Felt. *Chromosoma*. 1971; 33:421–435. [PubMed: 5569604]
- Gabrusewycz-Garcia N, Kleinfeld RG. A study of the nucleolar material in *Sciara coprophila*. *J Cell Biol*. 1966; 29:347–359. [PubMed: 5961345]
- Gauhar Z, Sun LV, Hua S, Mason CE, Fuchs F, Li T, Boutros M, White KP. Genomic mapping of binding regions for the ecdysone receptor protein complex. *Genome Res*. 2009; 19:1006–1013. [PubMed: 19237466]
- Gerbi SA, Liang C, Wu N, DiBartolomeis SM, Bienz-Tadmor B, Smith HS, Urnov FD. DNA amplification in DNA puff II/9A of *Sciara coprophila*. *Cold Spring Harb Symp Quant Biol*. 1993; 58:487–494. [PubMed: 7956063]
- Glover DM, Zaha A, Stocker AJ, Santelli RV, Pueyo MT, deToledo SM, Lara FJS. Gene amplification in *Rhynchosciara* salivary gland chromosomes. *Proc Natl Acad Sci USA*. 1982; 79:2947–2951. [PubMed: 6953439]

- Gonsalves SE, Neal SJ, Kehoe AS, Westwood JT. Genome-wide examination of the transcriptional response to ecdysteroids 20-hydroxyecdysone and ponasterone A in *Drosophila melanogaster*. *BMC Genomics*. 2011; 12:475. [PubMed: 21958154]
- Gronemeyer H, Pongs O. Localization of ecdysterone on polytene chromosomes of *Drosophila melanogaster*. *Proc Natl Acad Sci USA*. 1980; 77:2108–2112. [PubMed: 6769118]
- Gronemeyer H, Hameister H, Pongs O. Photoinduced bonding of endogenous ecdysterone to salivary gland chromosomes of *Chironomus tentans*. *Chromosoma*. 1981; 82:543–559. [PubMed: 7021084]
- Hackney JF, Pucci C, Naes E, Dobens L. Ras signaling modulates activity of the ecdysone receptor EcR during cell migration in the *Drosophila* ovary. *Dev Dyn*. 2007; 236:1213–1226. [PubMed: 17436275]
- Hartl T, Boswell C, Orr-Weaver TL, Bosco G. Developmentally regulated histone modifications in *Drosophila* follicle cells: initiation of gene amplification is associated with histone H3 and H4 hyperacetylation and H1 phosphorylation. *Chromosoma*. 2007; 116:197–214. [PubMed: 17219175]
- Kim JC, Orr-Weaver TL. Analysis of a *Drosophila* amplicon in follicle cells highlights the diversity of metazoan replication origins. *Proc Natl Acad Sci USA*. 2011; 108:16681–16686. [PubMed: 21933960]
- Kim JC, Nordman J, Xie F, Kashevsky H, Eng T, Li S, MacAlpine DM, Orr-Weaver TL. Integrative analysis of gene amplification in *Drosophila* follicle cells: parameters of origin activation and repression. *Genes Dev*. 2011; 25:1384–1398. [PubMed: 21724831]
- Kiss I, Benczw G, Fodor A, Szabad J, Fristrom J. Pupal larval mosaics in *Drosophila melanogaster*. *Nature*. 1976; 262:136–138. [PubMed: 819843]
- Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell*. 1991; 67:59–77. [PubMed: 1913820]
- Lara FJ, Stocker AJ, Amabis JM. DNA sequence amplification in sciarid flies: results and perspectives. *Braz J Med Biol Res*. 1991; 24:233–248. [PubMed: 1823237]
- Levine M. Paused RNA polymerase II as a developmental checkpoint. *Cell*. 2011; 145:502–511. [PubMed: 21565610]
- Liang C, Gerbi SA. Analysis of an origin of DNA amplification in *Sciara coprophila* by a novel three-dimensional gel method. *Mol Cell Biol*. 1994; 14:1520–1529. [PubMed: 8289825]
- Liang C, Spitzer JD, Smith HS, Gerbi SA. Replication initiates at a confined region during DNA amplification in *Sciara* DNA puff II/9A. *Genes Dev*. 1993; 7:1072–1084. [PubMed: 8504930]
- Lis JT. Imaging *Drosophila* gene activation and polymerase pausing in vivo. *Nature*. 2007; 450:198–202. [PubMed: 17994086]
- Lunyak VV, Ezrokhi M, Smith HS, Gerbi SA. Developmental changes in the *Sciara* II/9A initiation zone for DNA replication. *Mol Cell Biol*. 2002; 22:8426–8437. [PubMed: 12446763]
- Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR. DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science*. 2009; 324:407–410. [PubMed: 19372434]
- Monesi, N.; Candido-Silva, JA.; Paço-Larson, ML.; de Almeida, JC. Regulation of sciarid DNA puffs by ecdysone: mechanisms and perspectives. In: Smaggle, G., editor. *Ecdysone: structures and functions*. New York: Springer; 2009. p. 165-183.
- Pelling C. Ribonukleinsäure-Synthese der Riesenchromosomen Autoradiographische Untersuchungen an *Chironomus tentans*. *Chromosoma*. 1964; 15:71–122. [PubMed: 14171171]
- Poulson DF, Metz CW. Studies on the structure of nucleolus-forming regions and related structures in the giant salivary gland chromosomes of Diptera. *J Morphol*. 1938; 63:363–395.
- Rasch, EM. Two-wavelength cytophotometry of *Sciara* salivary gland chromosomes. In: Wied, GL.; Bahr, GF., editors. *Introduction to quantitative cytochemistry*. Vol. vol 2. New York: Academic; 1970a. p. 335-355.
- Rasch, EM. DNA cytophotometry of salivary gland nuclei and other tissue systems in dipteran larvae. In: Wied, GL.; Bahr, GF., editors. *Introduction to quantitative cytochemistry*. Vol. vol 2. New York: Academic; 1970b. p. 357-397.

- Robert M. Einfluss von Ionenstärke und pH auf die differentielle Dekondensation der Nukleoproteide isolierter Speicheldrüsen Zellkerne und Chromosomen von *Chironomus thummi*. *Chromosoma*. 1971; 36:1–33. [PubMed: 4945887]
- Rudkin G, Corlette SL. Disproportionate synthesis of DNA in a polytene chromosome region. *Proc Natl Acad Sci USA*. 1957; 43:964–968. [PubMed: 16590122]
- Stocker AJ, Pavan C. The influence of ecdysterone on gene amplification, DNA synthesis, and puff formation in the salivary gland chromosomes of *Rhynchosciara hollaenderi* (Diptera, Sciaridae). *Chromosoma*. 1974; 45:295–319. [PubMed: 4837973]
- Stocker AJ, Yokosawa J, Soares MA, Cadavid EO. DNA replication and amplification during the final cycle of polyteny in sciarid gland chromosomes and their control by ecdysone. *Ciênc Cult*. 1996; 48:306–312.
- Stocker AJ, Amabis JM, Gorab E, Elke C, Lezzi M. Antibodies against the D-domain of a *Chironomus* ecdysone receptor protein react with DNA puff sites in *Trichosia pubescens*. *Chromosoma*. 1997; 106:456–464. [PubMed: 9391218]
- Thomas HE, Stunnenberg HG, Stewart AF. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature*. 1993; 362:471–475. [PubMed: 8385270]
- Thummel CS. Puffs and gene regulation—molecular insights into the *Drosophila* ecdysone regulatory hierarchy. *Bioessays*. 1990; 12:561–568. [PubMed: 2127884]
- Urnov FD, Liang C, Blitzblau HG, Smith HS, Gerbi SA. A DNase I hypersensitive site flanks an origin of DNA replication and amplification in *Sciara*. *Chromosoma*. 2002; 111:291–303. [PubMed: 12474058]
- Wu N, Liang C, DiBartolomeis SM, Smith HS, Gerbi SA. Developmental progression of DNA puffs in *Sciara coprophila*: amplification and transcription. *Dev Biol*. 1993; 160:73–84. [PubMed: 8224550]
- Xie F, Orr-Weaver TL. Isolation of a *Drosophila* amplification origin developmentally activated by transcription. *Proc Natl Acad Sci USA*. 2008; 105:9651–9656. [PubMed: 18621687]
- Yao TP, Segraves WA, Oro AE, McKeown M, Evans RM. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell*. 1992; 71:63–72. [PubMed: 1327536]
- Yao TP, Forman BM, Jiang Z, Cherbas L, Chen JD, McKeown M, Cherbas P, Evans RM. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. *Nature*. 1993; 366:476–479. [PubMed: 8247157]
- Zink B, Engström Y, Gehring WJ, Paro R. Direct interaction of the Polycomb protein with Antennapedia regulatory sequences in polytene chromosomes of *Drosophila melanogaster*. *EMBO J*. 1991; 10:153–162. [PubMed: 1671215]

Fig. 1.

Western blots demonstrate the specificity of the ScEcR-A antibody. **a** Western blot with salivary gland extracts prepared from pre-amplification stages (pre-eyespot (*PES*), early eyespot (*EES*) and 8x4), amplification (10x5, 12x6), and post-amplification stages (14x7) were incubated with the polyclonal antibody to *Sciara* EcR-A. Arrow indicates a 68-kDa band corresponding to ScEcR-A (Foulk et al. 2013). Asterisk indicates a non-chromosomally bound cross-reacting band that is ~49 kDa. **b** Western blot of crude cytoplasmic and nuclear fractions of salivary glands. Labeled as described in **a**

Fig. 2. Localization of ScEcR-A on chromosome II at eyespot stage 8x4, 10x5, 12x6, and 14x7. The anterior one third portion of salivary glands was dissected from staged mid to late fourth instar *Sciara* larvae and squashed as described by Zink et al. (1991). Chromosome preparations were then reacted sequentially with a polyclonal antibody to ScEcR-A, Alexa Fluor 488 dye-conjugated goat anti-rabbit IgG, and counterstained with DAPI. **a, d, g, j** DAPI channel. **b, e, h, k** ScEcR-A (FITC) channel. **c, f, i, l** Merge of DAPI and ScEcR-A channels. *Yellow arrows* point to loci that form DNA puffs, *red arrows* point to loci that

form RNA puffs, and *white arrows* point to prominent examples of orphan signals (see text). *Bar* represents 50 μm

Fig. 3.
Localization of ScEcR-A on chromosome III at eyespot stage 8x4, 10x5, 12x6, and 14x7.
Preparation and details as described in Fig. 2

Chromosome Res. Author manuscript; available in PMC 2014 July 01.

Fig. 5.
Localization of ScEcR-A on chromosome X at eyespot stage 8x4, 10x5, 12x6, and 14x7.
Preparation and details as described in Fig. 2

Fig. 6.

Summary of ScEcR-A binding across the genome. Polytene chromosome maps are adapted from Gabrusewycz-Garcia (1964; with kind permission from Springer Science+Business Media B.V.). DNA and RNA puffs as listed in Gabrusewycz-Garcia (1964, 1971) and summarized in Table 1. **a** Chromosomes II, III, and IV at eyespot stage 10x5. **b** Chromosomes II, III, and IV at eyespot stage 14x7. Chromosome X was omitted because the triple repeat regions make analysis of ScEcR-A binding especially difficult. *Yellow text* in a black box denotes DNA puffs, *red text* indicates RNA puffs, and *black text* indicates prominent examples of ScEcR-A orphan signals (see text). *Green* indicates sites of ScEcR-A binding. *Bar* represents 10 μ m

Fig. 7.

Ecdysone injections into pre-amplification stage larvae prematurely induce the natural progression of ScEcR-A signals across the genome. Pre-amplification stage larvae were individually micro-injected with 320 pg of 20-hydroxyecdysone in 50 % ethanol. After injection, larvae were incubated at 21 °C and then used for chromosome squashes and ScEcR-A immunofluorescence experiments at the indicated times. Preparation of chromosome squashes and other details as described in Fig. 2. ScEcR-A immunostaining at **a** 12 h, **b** 15 h, and **c** 18 h after ecdysone injection

Table 1

Sciara coprophila salivary gland polytene chromosome puffs. Summary of ScEcR-A signals observed across the genome of *Sciara coprophila*

Chromosome	DNA puffs	RNA puffs	Prominent orphan signals
II	2B, 6A, 9A, 11A (post), (13A), (14B-post)	1B, 10B/C, 12A, 12B, 13C	4A
III	2B (ant), 10A, 11A, 15B (ant)	4A, 5C, 7A, 9B, 12C	13C
IV	(5C), (8C), (10B), (12A-ant), 15B (post), 19A	4C, 5B, 6B, 7B, 9C, 10A, 10C, 19C	14C
X	(7A), (11B)	4A, 4B, 8B, 13B, 14B, 14C, 15A, 15B, 16C, 17B	None

DNA and RNA puffs are listed as in Gabrusewycz-Garcia (1964, 1971). ant and post indicate that the puff occurs only in the anterior or posterior portion of the gland, respectively. DNA puffs in parentheses are minor puffs