Chapter 6
Regulation of Sciarid DNA Puffs by Ecdysone: Mechanisms and Perspectives

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Abstract DNA puffs, formed in the salivary gland polytene chromosomes at the end of the larval stage, are characteristic of Sciaridae. Cytological demonstrations coupled to the molecular characterization of DNA puffs revealed that they are sites of disproportional DNA synthesis and abundant transcription. The biological purpose of DNA amplification at these *loci* is to enable the synthesis of large amounts of proteins in short periods of time. Here, the role of ecdysone in DNA puffs formation and DNA puff genes amplification and expression is reviewed and a new model for ecdysone action on DNA puffs is proposed. Studies in transgenic *Drosophila* that contributed to the understanding of the mechanisms that regulate Sciaridae DNA puff gene amplification and transcription are described. Finally, the availability of antibodies raised against the EcR, will further extend the knowledge about the roles exerted by ecdysone in this unique model system.

Keywords DNA puffs • Sciaridae • gene amplification • transcription • ecdysone

6.1 The Discovery of DNA Puffs

The DNA puffs, a feature of sciarid polytene chromosomes, were initially reported by Breuer and Pavan (1952), while working with *R. americana* (called *R. angelae* in their investigation). They described a structure that forms in the polytene chromosomes of the salivary glands of fourth instar larvae that were similar to the

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"Balbiani Rings" observed in *Chironomidae*. In 1955, the same authors observed a disproportional increase in the DNA content of these puffs by Feulgen staining when compared to the neighboring bands (Breuer and Pavan, 1955). They interpreted this phenomenon as an adaptation for increased template production in these *loci*, which would result in the abundant synthesis of specific salivary gland polypeptides at the end of the fourth larval instar. Experimental evidence confirming that these regions constitute sites of disproportional DNA synthesis were obtained through the observation of higher [^{3}H]-thymidine incorporation in these puff regions (Ficq and Pavan, 1957) and with microspectrophotometric measurements of DNA (Rudkin and Corlette, 1957). DNA puffs are also *loci* of abundant RNA transcription. Experiments measuring [^{3}H]-uridine incorporation revealed high levels of RNA synthesis in the DNA puff forming regions at the time the puffs are expanded (Gabrusewycz-Garcia, 1968; Pavan and Da Cunha, 1969). Pavan (1965) called these puffs "DNA puffs" in order to distinguish them from the RNA puffs that occur in salivary gland polytene chromosomes of *Chironomus* and *Drosophila*. Similar studies in other sciarids such as *Sciara coprophila* (Crouse and Keyl, 1968; Gabrusewycz-Garcia, 1964; Rasch, 1970; Swift, 1962), *Sciara ocellaris* (Perondini, 1968), *Bradysia hygida* (Sauaia et al., 1971), and *Trichosia pubescens* (Amabis, 1974) confirmed that DNA puffs are sites of developmentally regulated differential DNA replication.

Formation of DNA puffs is dependent on the disproportionate synthesis of DNA (Breuer and Pavan, 1955). In this context, DNA puffs are targets of drugs that either inhibit DNA synthesis or modify the chemical properties of DNA. Three drugs, that act through distinct mechanisms, were extensively employed in *B. hygida* (Fig. 6.1): hydroxyurea (HU) (Sauaia et al., 1971), 2,3-dihydro-1-H-imidazo (1,2-b) pyrazole (IMPY) (Ribeiro, 1975) and the thymidine analog 5-bromodeoxyuridine (BrdUrd) (de Almeida, 1978). The age when the process of gene amplification is beginning (E3) is named the "critical time", and the injection of either HU or IMPY at this age completely prevented the formation of the two groups of DNA puffs in *B. hygida* (Fig. 6.1). The inhibition of DNA puff formation occurred without affecting the pattern of RNA puffs formation and without impairing larval development (Ribeiro, 1975; Sauaia et al., 1971). In the case of BrdUrd, the injection between 18 and 10 h before E7 resulted in modifications of the chromatin structure of the DNA puff anlages and impaired the expansion of DNA puffs, but did not interfere either with the activity of the RNA puffs, or the larval development (de Almeida, 1978).

6.2 Effect of 20-Hydroxyecdysone on DNA Puff Formation

Crouse (1968) carried out the first experiments demonstrating that DNA puff formation and DNA amplification are both regulated by ecdysone. The injection of ecdysone in young larvae of *S. coprophila* induced DNA amplification at the DNA puff forming sites 24 h after the injection (Crouse, 1968). In *Rhynchosciara angelae,* experiments in which salivary glands of young larvae were implanted into the body cavity of older larvae led to extra synthesis of DNA in the salivary gland

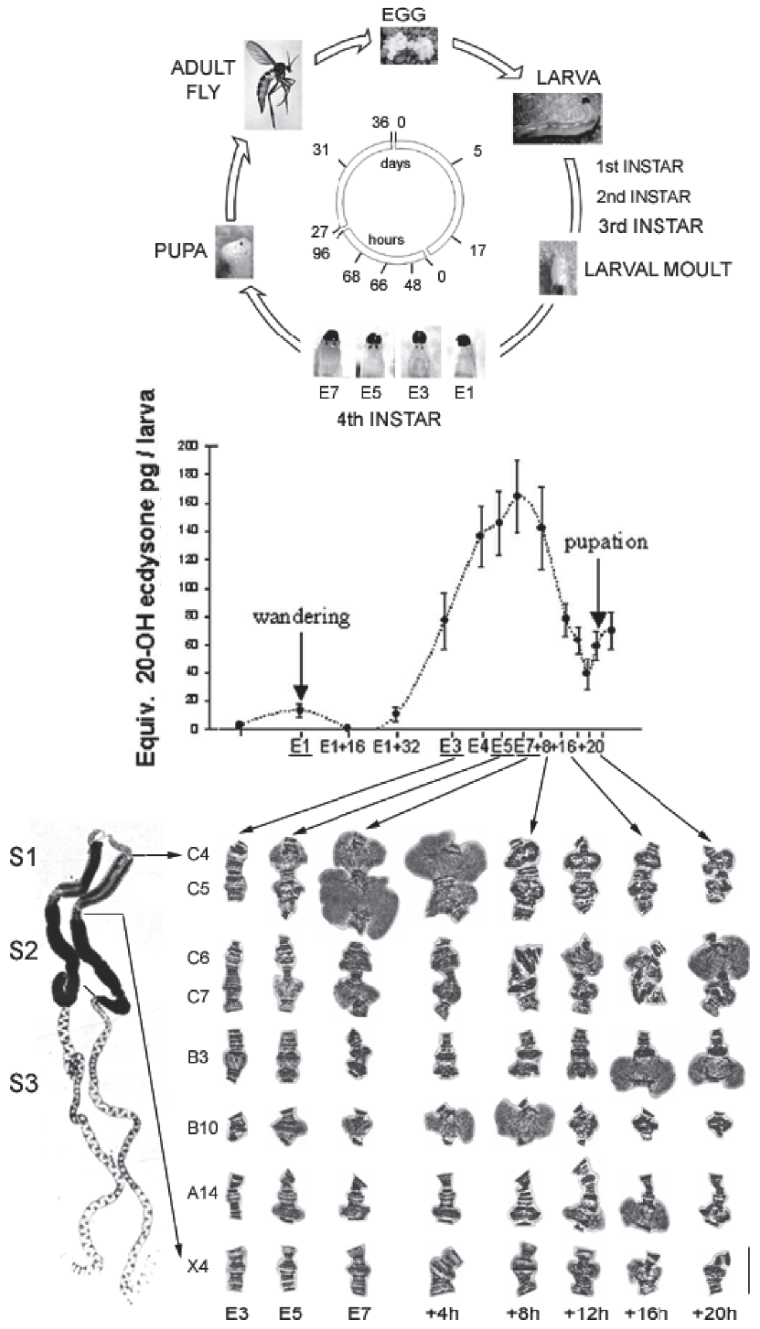

Fig. 6.1 *B. hygida* life cycle, ecdysone titers and DNA puffs expansion and regression during the end of the fourth larval instar. The sciarid *B. hygida* started to be cultured in 1965 (Sauaia and Alves, 1968). *Top*, *B. hygida* life cycle. In the laboratory, at 20°C, *B. hygida* life cycle lasts about 36 days. The embryonic stage lasts 5 days, from egg laying to hatching of first instar larvae. The

chromosome, of both the host and the implanted gland. The implantation of salivary glands of old larvae into young larvae led to the regression of the DNA puffs in the implanted salivary gland chromosomes (Amabis and Simoes, 1971).

Further experiments in *Rhynchosciara* demonstrated that DNA amplification and DNA puff formation are both observed 24 h after ecdysone injection (Alvarenga et al., 1991; Berendes and Lara, 1975; Fresquez, 1979; Stocker and Pavan, 1974) and is a process dependent on RNA synthesis (Alvarenga et al., 1991; Berendes and Lara, 1975). Similar results were also obtained after the injection of ecdysone in *T. pubescens* larvae which revealed that the effect of ecdysone on DNA synthesis and DNA puff formation are both a late response to the hormone (Amabis and Amabis, 1984a). Studies in *T. pubescens* demonstrated that once amplification has started, it will continue even if the hormone source is eliminated by ligature of the larvae. In this case, DNA amplification occurs in the absence of DNA puff formation, showing that higher titers of hormone are required for DNA puff formation (Amabis and Amabis, 1984b).

The role of ecdysone on RNA transcription in DNA puffs was also investigated through ligature experiments in *T. pubescens* (Amabis and Amabis, 1984b). In these

Fig. 6.1 (continued) larval stage comprises four instars. The fourth larval instar begins on the 17th day after egg laying. At the 6th day of the fourth instar (23rd day after egg laying) the eyespots arise (age E1, 0 h) which are useful staging landmarks. The pupal molt occurs on the 27th day, metamorphosis lasts 4 days which is terminated by the emergence of the adults. *Middle*, results from the measurements by radioimmunoassay (RIA) of 20-hydroxyecdysone titers in the hemolymph of *B. hygida* larvae during the end of the fourth larval instar (reproduced from Basso et al., 2002). Mean values and standard errors represent measurements of at least four animals in each developmental stage. The small peak at stage E1 (commitment peak), corresponds to a 15-fold increase in 20-hydroxyecdysone titers and the second and broader peak at stage E7 corresponds to a 200-fold increase over basal hormone levels. A decay in the ecdysone levels was observed before the onset of pupation, after which the hormone levels start to rise again. *Bottom left* shows a pair of fourth instar salivary glands (reproduced from Laicine et al., 1984). The salivary glands are about 10 mm long and consist of two rows of cells and three morphologically distinct regions: S1 (anterior), S2 (granulose) and S3 (posterior). About 190 cells form each gland (Paçó-Larson, 1976; Sauaia et al., 1971), and about 45 of them are in the S1 region. All DNA puffs (*bottom right*) are formed in the S1 and S3 regions with the exception of DNA puff C7 which is only formed in the S1 region (reproduced from Laicine et al., 1984). The first group of DNA puffs does not form in the S2 region, and the puffs of the second group are very inconspicuous in this gland region (Sauaia et al., 1971). The eyespot E3 (48 h after E1) coincides with the beginning of the process of gene amplification and DNA puff anlage formation at several chromosomal sites in salivary gland polytene chromosomes. There are eight major DNA puff forming regions, whose expansion is finely regulated throughout development and which can be classified in two distinct groups. At stage E5, the first group of DNA puffs starts its expansion (DNA puffs C7, C5 and C4), 2 h latter the E7 pattern is attained, when these three DNA puffs are open and about 8 h latter the first group of DNA puffs has already closed. The expansion of the second group of DNA puffs, puffs A14, B3d, C6 and X4, starts about 12 h after E7, and before the pupal molt (E7 + 26 h) these puffs are already closed, with the exception of puff B3d. The DNA puff B10 is the only DNA puff active between the periods of expansion of the first and second groups of DNA puffs and its maximum size is attained at E7 + 8 h (Laicine et al., 1984)

experiments, the ligature blocked the release of ecdysone in the posterior region of the larvae and transcriptional activity was assessed by incubation of salivary glands in medium containing ^{3}H-uridine. In the anterior region large DNA puffs were formed which displayed a heavy ^{3}H-uridine labeling, whereas in the posterior region DNA puffs were not formed and the labeling was uniformly distributed (Amabis and Amabis, 1984b). Together, these results constitute cytological demonstrations that ecdysone treatment induces DNA amplification, DNA puff formation and RNA transcription at DNA puff forming regions. These observations are in agreement with the ecdysone titer measurements which have been performed in fourth instar larvae of both *R. americana* (Stocker et al., 1984) and *B. hygida* (Fig. 6.1) (Basso et al., 2002).

6.3 DNA Puffs and Protein Synthesis

The expansion and regression of specific DNA puffs can be related to the developmentally regulated synthesis of groups of salivary gland polypeptides (de Almeida, 1997; Laicine et al., 1984; Winter et al., 1977). In *B. hygida*, the pattern of protein synthesis in the salivary gland during the fourth larval instar was carefully investigated (Laicine et al., 1984). During the first 9 days of the fourth instar, the three salivary gland regions synthesize characteristic sets of polypeptides (first period of protein synthesis). After this period of sustained protein synthesis, the S1 and S3 salivary gland regions carry out two successive, new programs of synthesis (second and third periods of protein synthesis), which last about 12 h each. The striking temporal coincidence, between the expansion of the two groups of DNA puffs (Fig. 6.1) and the second and third periods of protein synthesis, respectively, suggested that these newly synthesized proteins were the products of the DNA puffs, which were produced during short periods of time while the puffs were expanded (Laicine et al., 1984). Studies in *R. americana* and *T. pubescens* reached similar conclusions (Amabis and Janczur, 1978; de Toledo and Lara, 1978; Winter et al., 1977) and indicated a causal relationship between DNA puffs and the synthesis of specific proteins.

In *B. hygida*, the selective inhibition of DNA puffs development in both the S1 and S3 salivary gland regions, by either HU, or IMPY or BrdUrd, is accompanied by the inhibition of the same polypeptides characteristic of the second and third periods of synthesis, without affecting those produced during the first period of synthesis (Laicine et al., 1982, 1984). These results revealed that both DNA puff formation and DNA synthesis, processes triggered at E3, are essential for the correct reprogramming of protein synthesis in the later stages (Laicine et al., 1984). These results agree with those obtained both in *R. americana* (Bonaldo et al., 1979; Winter et al., 1980) and *T. pubescens* (Amabis, 1980) and led to the suggestion that the biological purpose of DNA amplification is to enable the synthesis of large amounts of proteins in short periods of time. The process of developmentally regulated gene amplification was probably selected in order to fulfill a demand that would probably not be attained by standard gene expression mechanisms (Laicine et al., 1984).

The pattern of DNA puff protein synthesis in *B. hygida* was extended through the analysis of both the patterns of global protein synthesis and the characterization of the patterns of secreted polypeptides. Radiometry measurements of the saliva of larvae injected with ^{3}H-leucine revealed that most of the cumulative secretion of radioactive proteins occurs during the period of DNA puffs activity and reaches maximum levels of synthesis at age E7 + 6 h, at amounts that correspond to 28.5 times the amount of protein secreted at age E1 (de Almeida, 1997). The characterization of *B. hygida* salivary gland extracts by fluorography revealed a dynamic pattern of polypeptide synthesis in the salivary gland at the time of DNA puff formation. Correlations drawn between the time of appearance of some polypeptides and the formation of DNA puffs led to the suggestion that a 43 and a 23 kDA polypeptide constitute the products of DNA puff C4 and B10, respectively (de Almeida, 1997). These results were latter confirmed by the demonstration that polyclonal antibodies raised against either the product of DNA puff B10, the BhB10-1 protein, or against the product of DNA puff C4, the BhC4-1 protein, detect the same polypeptides as previously identified by fluorography (Fontes et al., 1999; Monesi et al., 2004).

Several DNA puff genes have been cloned and searches with full length cDNAs against the public databases revealed that the DNA puffs polypeptides have no orthologs in the complete genome sequences of four other insect species (*Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera* and *Tribolium castaneum*). Based on the similarity of the deduced amino acid sequences three DNA puff protein families have been identified: family I comprises the product of DNA puff C3 of *R. americana*, the II/9-1 and II/9-2 proteins of DNA puff II/9A of *S. coprophila* and the product of DNA puff C-4B of *T. pubescens*; family II comprises the BhC4-1 protein from DNA puff C4 of *B. hygida*, and the products of both DNA puff C8 and B2 of *R. americana*; family III comprises the BhB10-1 protein of DNA puff B10 of *B. hygida* (Penalva et al., 1997; Santelli et al., 2004).

6.4 The Role of Ecdysone in the Reprogramming of Protein Synthesis at the End of the Fourth Larval Instar

The injection of ecdysone in young larvae (E1) induced both the second and third periods of protein synthesis in the salivary gland (de Carvalho DP and de Almeida JC, 1993). These results were confirmed by the direct demonstration that the BhC4-1 protein, a member of the second period of protein synthesis, is detected in salivary gland extracts 24 h after the injection of hormone in larvae at E1, and indicated that the expression of this protein is a late response to the hormone (Basso et al., 2002) (Fig. 6.2). When salivary glands at the age E1 were cultivated *in vitro* in the presence of the hormone, neither the second nor the third period of protein synthesis were induced, indicating that some other factor, besides the hormone, is necessary to promote DNA puff genes expression (de Carvalho DP and de Almeida JC, 1993). These results are in agreement with those obtained in *Rhynchosciara*, which revealed that the complete induction of DNA puff B2

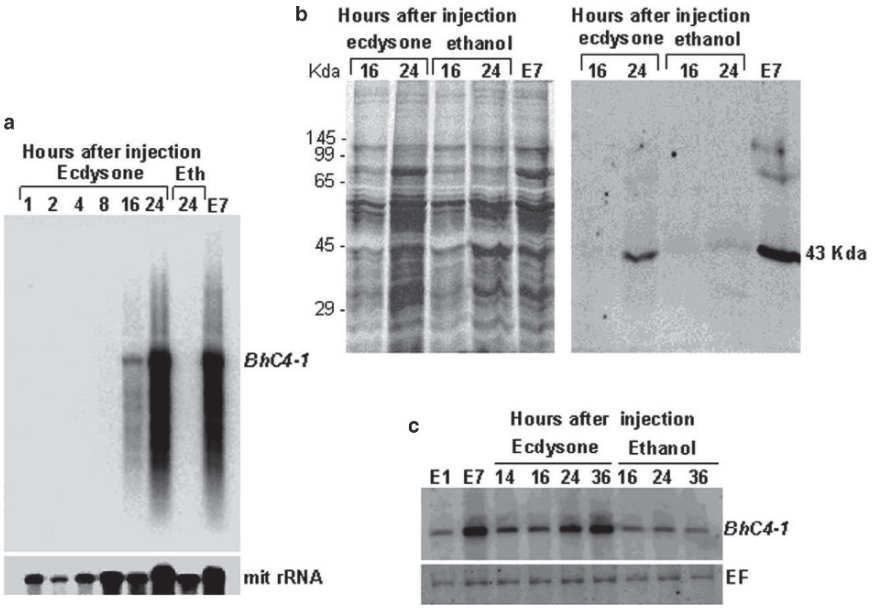

Fig. 6.2 *BhC4-1* expression and amplification are both induced by ecdysone (Reproduced from Basso et al., 2002). (**a**) Northern blot containing 20 µg of total RNA extracted from salivary glands of larvae injected at stage E1 with 0.4 mM ecdysone and dissected at different times after injection (1, 2, 4, 8, 16 and 24 h), 20 µg of total RNA extracted from salivary glands of larvae injected with 5% ethanol and dissected 24 h latter (Eth/24) and 20 µg of total RNA extracted from salivary glands of larvae at stage E7. The upper autoradiogram shows the result after hybridization with a *BhC4-1* cDNA fragment and the bottom autoradiogram shows the result after hybridization of the same blot with a 500 bp fragment from a cDNA encoding the mitochondrial large rRNA subunit of *Anopheles gambiae* (Lemos et al., 1996). (**b**) *Left hand picture*, Coomassie Blue stained SDS-PAGE (9%) of total salivary gland extracts of larvae injected at stage E1 with ecdysone or with 5% ethanol, and dissected at the indicated times and total salivary gland extracts of larvae at stage E7. *Right hand picture*, immunoblot of an identical gel after incubation with an affinity-purified anti-BhC4-1 antibody followed by detection with chemiluminescence. The 43 kDa polypeptide is the BhC4-1 product (Monesi et al., 2004). (**c**) *Upper autoradiogram*: southern blot containing 10 µg of *Eco* RI digested DNA extracted from salivary glands of larvae at stage E1 and E7, 10 µg of *Eco* RI digested DNA from salivary glands of larvae injected at stage E1 with 0.4 mM ecdysone and dissected at different times after injection (14, 16, 24 and 36 h) and 10 µg of *Eco* RI digested DNA from salivary glands of larvae injected at stage E1 with 5% ethanol and dissected at different times after injection (16, 24 and 36 h), after hybridization with a 1.2 kb *BhC4-1* cDNA fragment which detects a 9 kb *Eco* RI genomic fragment. *Bottom autoradiogram*, the blot was stripped, followed by hybridization with a cDNA fragment encoding the elongation factor 1α (EF-1α) of *R. americana* (Graessmann et al., 1992)

activity *in vitro* was only attained when salivary glands were cultivated in the presence of both the hormone and hemolymph (Alvarenga, 1980).

In older *B. hygida* larvae (age E7), the presence of the hormone in the culture medium was sufficient to sustain the synthesis of the polypeptides characteristic of the second period of synthesis, and inhibited the appearance of the polypeptides

of the third period of synthesis (de Carvalho et al., 2000). Furthermore, the injection of hormone in larvae at E7 + 10 h, developmental time when the polypeptides characteristic of the third period of synthesis are induced, inhibited the synthesis of this group of polypeptides (de Carvalho et al., 2000). These results indicate that the two groups of DNA puffs of *B. hygida* respond to different thresholds of hormone: the expression of the genes of the first group of DNA puffs (second period of protein synthesis) depend on the presence of high titers of hormone, whereas the expression of the genes of the second group of DNA puffs (third period of protein synthesis) occurs in the presence of lower titers of hormone (de Carvalho et al., 2000).

These results, together with direct measurements of 20-hydroxyecdysone concentration in the hemolymph of larvae during the end of the fourth instar (Basso et al., 2002), do not fit the model described by Lara et al., (1991) which proposed that high levels of hormone are necessary during the entire period of gene amplification and DNA puff expansion. The current model on the ecdysone action on DNA puffs, derived from studies in *B. hygida*, proposes that the high ecdysone titers in the hemolymph at E5 (Fig. 6.1) induce the expansion of the first group of DNA puffs and the expression of the polypeptides characteristic of the second period of protein synthesis (Basso et al., 2002; de Carvalho et al., 2000). During cocoon spinning (late E7), the levels of hormone and probably its receptor, start to decrease, which results in reduced expression levels of the amplified genes and the regression of the first group of DNA puffs. At age E7 + 12, when the hormone titers are lower, the expression of the genes of the second group of DNA puffs is induced (third period of protein synthesis) (Basso et al., 2002; de Carvalho et al., 2000). At the end of the fourth larval instar, the increase in the 20-hydroxyecdysone titers (Fig. 6.1) results in the down regulation of the genes of the second group of DNA puffs prior to the larvae to pupae transition and to the transformations typical of metamorphosis, including elimination of the salivary gland by a process of programmed cell death (Simon and de Almeida, 2004).

6.5 Mechanism of Gene Amplification in DNA Puffs

Gene amplification in DNA puff forming regions results from re-replication events triggered in specific regions of the salivary gland chromosomes. The amplified DNA accumulates in the salivary glands (Fontes et al., 1992; Paçó-Larson et al., 1992; Wu et al., 1993; Yokosawa et al., 1999) and remains bound to the DNA puff forming sites (Paçó-Larson, 1982; Rasch, 1970) until at least the end of the larval stage. Amplification levels of between 4- to 30-fold were demonstrated depending on the DNA puff analyzed (Fontes et al., 1992; Glover et al., 1982; Monesi et al., 1995; Paçó-Larson et al., 1992; Santelli et al., 2004; Wu et al., 1993). The DNA strands synthesized during amplification are arranged in parallel (Fontes et al., 1992; Paçó-Larson et al., 1992; Santelli et al., 1991; Wu et al., 1993), as opposed to the

arrangement *in tandem* demonstrated for the amplicons formed as part of the drug resistance response in mammalian cells (Ma et al., 1988). Quantitative hybridization studies performed in *B. hygida* (Coelho et al., 1993; Monesi et al., 1995) and in *R. americana* (Stocker et al., 1996; Yokosawa et al., 1999) indicated the existence of an amplification gradient in the DNA puff regions similar to that described for the amplicons formed in the ovarian follicular cells of *D. melanogaster* (Claycomb et al., 2004; Spradling, 1981). The fragments containing the genes whose temporal pattern of expression are related to DNA puffs expansion reach higher amplification levels than fragments containing sequences expressed when the puff is absent (Coelho et al., 1993; Monesi et al., 1995).

In the *Drosophila* chorion *loci*, the high frequency of replication initiation, coupled to low elongation speed, results in an amplification gradient that extends over approximately 100 kb and is centered on an origin of replication region which is repetitively triggered in the follicular cells at the end of the oogenesis (for review, Orr-Weaver, 1991). The amplification mechanism in the chorion *loci* was further characterized by electron microscopy studies of chromatin spreading (Osheim et al., 1988), which revealed a structure of forks within forks, named "onion skin", and through bi-dimensional electrophoreses analysis of replication intermediates (Delidakis and Kafatos, 1989; Heck and Spradling, 1990). Quantitative hybridization results in *B. hygida* (Coelho et al., 1993; Monesi et al., 1995), coupled to the identification of bi-directional origins of replication which are active during the amplification period in both *S. coprophila* DNA puff II/9 (Liang et al., 1993) and DNA puff C3 of *R. americana* (Yokosawa et al., 1999), led to the proposal that the amplification in DNA puffs forming sites also occurs by an onion skin type mechanism. In these *loci*, replication bubbles were detected upstream of the transcription initiation site of the genes whose expression is temporally related to the respective puff expansion (Liang et al., 1993; Yokosawa et al., 1999). Similarly, the *BhC4-1* gene of the *B. hygida* DNA puff C4 is amplified by forks that emanate from its 5′ region, although in this case the origin of replication region has not been cloned (Coelho, 1997).

The characterization by bi and tri-dimensional electrophoresis of the replication zone in DNA puff II/9A of *S. coprophila* revealed that each DNA strand is replicated from an unique site within a ~1 kb initiation region (ORI), located ~2 kb upstream of the *II/9A-1* gene transcription initiation site (Liang and Gerbi, 1994; Liang et al., 1993). Immediately upstream of the DNA puff II/9 ORI region lies a 80 bp fragment, which binds the origin replication complex (ORC). The binding of ORC to this 80 bp fragment is ATP dependent (Bielinsky et al., 2001). These results demonstrated that the binding of ORC to DNA fragments adjacent to the replication initiation site also occurs in metazoans (Diffley et al., 1994). The ORI region in the *S. coprophila* DNA puff II/9 is part of an initiation region employed during both the salivary gland endocycles and the embryonic mitotic cycles, indicating that the DNA replication machinery employed in cell proliferation and growth is also used in amplification (Lunyak et al., 2002). The results obtained in sciarids agree with those derived from the molecular characterization of mutations associated with the thin eggshell phenotype in *Drosophila* (Claycomb and Orr-Weaver, 2005). The demonstration that intrachromosomal amplification recruits part of the conserved

replication initiation machinery indicates the existence of a mechanism that specifies these amplified *loci* for re-replication. The understanding of this mechanism is a goal that is being pursued both in *Drosophila* follicular cells and in sciarid DNA puffs.

A ~400 bp DNase hypersensibility region (DHS), which is more susceptible to endonuclease clevage during the amplification period than after amplification, was mapped downstream of the DNA puff II/9A ORI of *S. corpophila* (Urnov et al., 2002). This DHS may constitute a DNA puff amplification regulator similar to the ACEs (Amplification Control Element) formerly described in the *Drosophila* chorion genes *loci*. The ACEs are defined as elements able to induce DNA amplification when inserted in different genomic sites independently of their orientation and distance (Orr-Weaver et al., 1989; Zhang and Tower, 2004). The sequence of the ACE3 element characterized in *D. melanogaster* is conserved between various species of *Drosophila* (Swimmer et al., 1990). Heterologous systems were employed to investigate the regulation of DNA puff amplification and/or gene expression (Bienz-Tadmor et al., 1991; Millar et al., 1985; Monesi et al., 1998). The most conclusive study was obtained by the analysis of transgenic *D. melanogaster* lines carrying an 18 kb fragment of the DNA puff C3 of *R. americana* that contained the ORI region active during amplification (Yokosawa et al., 1999) and which was flanked by su(Hw)BS elements, to minimize possible position effects (Lu and Tower, 1997). The absence of *R. americana DNA* puff amplification in both the salivary gland and in the follicular cells in the *Drosophila* transgenic lines indicated that factors involved in DNA puff C3 amplification are not conserved between the sciarids and *Drosophila* (Soares et al., 2003).

Sequence searches performed in the NCBI non-redundant database revealed 90% identity in a 72 bp sequence present both in the *S.coprophila* DNA II/9A DHS sequence and in the ORI region of *R. americana* DNA puff C3 (Fig. 6.3), and suggested a role for this 72 bp sequence in DNA puffs amplification. The absence of identity between the DNA II/9A DHS sequence and the *Drosophila* ACEs is not surprising and agrees with the results derived from functional studies, which

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DHS-Sci  202 AACACGAGGCGAATAAAAAGTGCATCGTTCTAAGCCGAATTTTAAAAGACAGAC---AAT 258
             ||||||||| |||||||| |||||||| |||||||||||||| ||||||||||   |||
ORI-Rhy 5889 AACACGAGGTGAATAAAA-GTGCATCGGTCTAAGCCGAATTTT-AAAGACAGACTTAAAT 5946

DHS-Sci  259 ATTTACACAGTTTGC 273
             |||||||||||||||
ORI-Rhy 5947 ATTTACACAGTTTGC 5961
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Fig. 6.3 The *R. americana* DNA puff C3 ORI region share a 71–72 bp sequence displaying 90% identity with the DNase Hypersensitive Site (DHS) adjacent to the ORI of *S. coprophila* DNA II/9A amplicon. The alignment is the result of a Blastn of the DHS 419 pb sequence of *S. coprophila* against the NCBI non redundant databank (NCBI accession numbers:AY225408, *R. americana* and AF464926, *S. coprophila*)

suggested that the factors that regulate intra-chromosomal DNA amplification during the normal course of development are not conserved between sciarids and *Drosophila* (Soares et al., 2003).

6.6 The Role of Ecdysone in DNA Puff Gene Amplification

In *B. hygida*, the selective inhibition of DNA puffs development by HU at E3 ("critical time"), suppresses the formation of DNA puff anlage (Sauaia et al., 1971) and indicates that the re-replication involved in the amplification process begins simultaneously at the DNA puffs forming regions. The fact the "critical time" defined in *B. hygida* coincides with the increase in ecdysone titers in the hemolymph (Fig. 6.1), agrees with the idea that the hormone is the developmental cue that triggers amplification in all DNA puffs forming sites. Accordingly, the injection of ecdysone in larvae during the pre-amplification stage, when the ecdysone levels are low, induces the amplification of *B. hygida* DNA puff *BhC4-1* gene (Basso et al., 2002) (Fig. 6.2). Similar results were also recently described for the II/9A *locus* of *S. coprophila* both *in vivo* and *in vitro*. In *in vitro* experiments, the addition of transcription and protein synthesis inhibitors to the culture medium prevented the induction of amplification and suggested an indirect mechanism for the action of ecdysone in amplification (Foulk et al., 2006).

Immunolocalization experiments in polytene chromosomes of *T. pubescens*, using a polyclonal antibody raised against the EcR of *Chironomus*, revealed that the EcR is bound in DNA puff-forming *loci* before and during DNA puffs expansion (Stocker et al., 1997). More recent experiments, using polyclonal antibodies developed against the EcR of *B. hygida*, revealed the presence of the receptor in all *B. hygida* DNA puffs when they are expanded (Fig. 6.4 and Candido-Silva et al.,

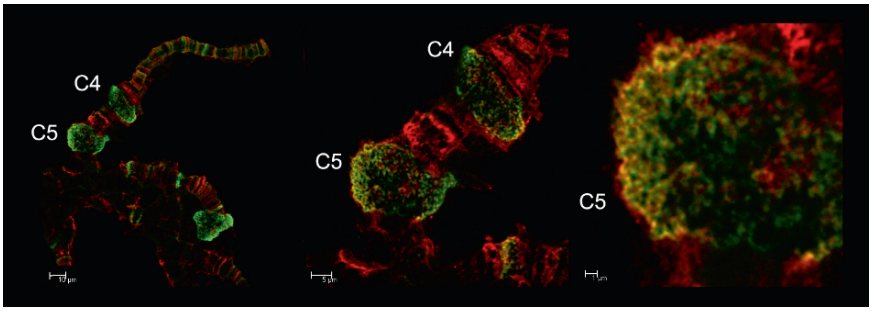

Fig. 6.4 The BhEcR colocalizes with RNA polymerase II at DNA puff forming sites. Chromosome C from *Bradysia hygida* larvae at age E7 labeled with antibodies anti-BhEcR (red) and anti-RNA Pol II (green). Yellow signals indicate colocalization of both antibodies. From left to right, images of the same field were captured by confocal microscopy at increasing magnifications. The bars at the bottom left of each picture correspond, from left to right, to 10 µm, 5 µm and 1 µm, respectively (*See Color Plates*)

2008). Furthermore, electrophoretic mobility shift assays (EMSA) revealed that a putative ecdysone receptor binding site, localized between the *S. coprophila* II/9A DHS sequence and the ORC binding region, binds the *Sciara* EcR efficiently, perhaps suggesting that the EcR could be the elusive amplification factor (Foulk et al., 2006). At present, it is difficult to define whether the EcR/USP receptor exerts its influence by modulating amplification, transcription, or both processes. The function of the EcR in DNA puff forming sites might be broader. Remarkably, alterations in the histone acetylation in the ORC binding sites of *Drosophila* chorion genes interfere with chorion genes amplification (Aggarwal and Calvi, 2004). In this sense, it is possible that the EcR acts as an anchor to recruit chromatin modulator factors, which could in turn create an environment favorable for the binding of other factors that modulate the functions of DNA puffs.

6.7 The Role of Ecdysone on the Transcription of DNA Puff Genes

Sciarid DNA puffs are sites of developmentally regulated transcription (Coelho et al., 1993; DiBartolomeis and Gerbi, 1989; Fontes et al., 1992; Frydman et al., 1993; Glover et al., 1982; Monesi et al., 1995; Paçó-Larson et al., 1992; Penalva et al., 1997; Santelli et al., 1991; Santelli et al., 2004; Wu et al., 1993). *In vitro* experiments revealed that the presence in the culture medium of other factor(s), besides the hormone, that was or were necessary in order to fully induce DNA puff genes expression (Alvarenga, 1980; de Carvalho et al., 2000; Foulk et al., 2006). In *R. americana*, the expression of the B-2 mRNA in the salivary gland was induced 24 h after ecdysone injection (Alvarenga et al., 1991; Bonaldo et al., 1979; de Toledo and Lara, 1978). In *B. hygida* maximum levels of *BhC4-1* mRNA expression are detected in the salivary gland when the ecdysteroid titers in the hemolymph are highest and the injection of ecdysone in larvae at (E1) induces *BhC4-1* mRNA in the salivary gland 16 h later (Basso et al., 2002; Paçó-Larson et al., 1992) (Fig. 6.2). More recent results in *S. coprophila* have shown that the *II/9-1* gene of DNA puff II/9A is also induced in the salivary gland 30 h after hormone injection (Foulk et al., 2006). Taken together, ecdysone injection experiments in sciarids revealed that the induction of DNA puff genes expression in the salivary gland occurs between 16–36 h after hormone injection, and constitute a late response to elevated levels of hormone in the hemolymph (Alvarenga et al., 1991; Basso et al., 2002; Foulk et al., 2006). In all cases, the complete induction of DNA puff genes expression has only been attained in *in vivo* experiments and depends on the injection of hormone (Alvarenga et al., 1991; Basso et al., 2002; Foulk et al., 2006).

In both *T. pubescens* and *B. hygida*, most of the chromosomal regions in which the EcR is detected also correspond to regions in which RNA polymerase II is bound (Candido-Silva et al., 2008; Stocker et al., 1997). All three EcREs (ecdysone response elements) identified in the *II/9-1* promoter region are able to bind protein complexes present in salivary gland nuclear extracts, which are supershifted in the

presence of an antibody specific to DmEcR (*Drosophila melanogaster* ecdysone receptor) (Foulk et al., 2006). Together, the results obtained in sciarids suggests that the EcR receptor might act as a *trans*-activating factor in the transcriptional regulation of DNA puff genes (Candido-Silva et al., 2008; Foulk et al., 2006; Stocker et al., 1997).

The characterization of DNA puff genes transcription regulatory mechanisms has been extended through functional assays in transgenic *Drosophila*. These studies revealed that the mechanisms that control the tissue specific developmentally regulated expression of both the *II/9-1* gene of *S. coprophila* and the *BhC4-1* gene of *B. hygida* are conserved in *Drosophila* (Bienz-Tadmor et al., 1991; Monesi et al., 1998). However, in transgenesis the DNA puff *BhB10-1* of *B. hygida* and the *C3-22* gene of *R. americana* are constitutively expressed at low levels throughout development (Monesi et al., 2001; Soares et al., 2003). Together, these results reveal that the conservation of DNA puff genes transcriptional regulatory mechanisms is not observed for all DNA puff genes.

In transgenic *Drosophila*, a 718 bp fragment from the *II/9-1* promoter of *S. coprophila* drives the expression of the CAT (chloramphenicol acetyltransferase) reporter gene in late prepupae. CAT activity is initially detected in 6 h prepupae and reaches maximum levels in 9 h prepupae (Bienz-Tadmor et al., 1991). The *B. hygida BhC4-1* mRNA is initially detected during the larvae to prepupae transition in transgenesis, and reaches its highest levels of expression in 3 h prepupae (Basso et al., 2002; Monesi et al., 1998). These results indicate that the *II/9-1* gene and the *BhC4-1* gene are under different temporal regulation in *Drosophila*. While the *BhC4-1* gene has been shown to be induced in response to the increase of hormone levels that trigger the larval to prepupal transition, the *II/9-1* gene is most likely induced in response to the prepupal peak of hormone that promotes the prepupae to pupae transition in *Drosophila*.

In *Drosophila*, it is possible to reproduce *in vitro* the entire sequence of RNA puffing when intermolt salivary glands are cultivated in the presence of ecdysone (Ashburner, 1972; Ashburner et al., 1974; Huet et al., 1993, 1995). In transgenesis, the induction of *BhC4-1* mRNA is a late response to the hormone that occurs only after 6 h exposure of salivary glands to the hormone, which is reminiscent of the response of *B. hygida* larvae after hormone injection (Basso et al., 2002). The incubation of transgenic salivary glands in the presence of cycloheximide in the absence of ecdysone also resulted in the induction of *BhC4-1* expression, indicating the participation of a repressor whose synthesis is necessary to maintain the gene repressed (Basso et al., 2002). A different situation has been observed for the *II/9-1* gene of *S. coprophila*. Whereas the injection of hormone in *S. coprophila* induces the *II/9-1* gene expression 30 h after injection (Foulk et al., 2006), in transgenesis the reporter gene expression driven by the *II/9-1* promoter was rapidly induced by ecdysone (Bienz-Tadmor et al., 1991). The early response of the *II/9-1* gene in transgenesis could be explained if the regulation of this gene also requires a repressor. If the tested *II/9-1* promoter fragment did not include the repressor binding site, or if the repressor is not present in *Drosophila*, an early induction of the gene would occur (Basso et al., 2002).

At the larvae to prepupae transition in *D. melanogaster* the ecdysone heterodimeric receptor EcR/USP (ultraspiracle) bound to ecdysone directly activates the expression of a small number of early response genes. The products of early genes are transcriptional regulators whose function is to induce a large set of late response genes (Thummel, 1996). As discussed above, both in *B. hygida*, and in transgenesis, the *BhC4-1* gene is induced as a late response to the increase in ecdysone titers which precede the metamorphosis (Basso et al., 2002). The role of the *Drosophila* early genes, *BR-C*, *E74* and *E75* on the regulation *BhC4-1* genes was recently investigated in *Drosophila* transformed with a chimeric *BhC4-1-lacZ* gene, through a loss of function approach, coupled to overexpression experiments (Basso et al., 2006). The results demonstrated that the product of the *BR-C* early gene, the BR-C Z3 isoform, is essential for the induction of *BhC4-1-lacZ* in the salivary gland and also revealed that the early gene products BR-C Z1, BR-C Z4, E75A, E74A and E74B participate to a lesser degree in the regulation of *BhC4-1-lacZ*. Since in the absence of the early gene products neither the developmental time nor the tissue specificity of *BhC4-1-lacZ* expression was affected, it has been suggested that the role of the early gene products is to regulate the correct levels of *BhC4-1* expression in the salivary gland at the larvae to prepupae transition (Basso et al., 2006). These results constitute the first demonstration that *trans*-activating factors, members of the ecdysone regulatory pathway, participate in the transcriptional regulation of a DNA puff gene and suggest that this regulatory pathway is conserved between *D. melanogaster* and *B. hygida*.

6.8 Future Perspectives

The last decade has witnessed important discoveries regarding the role of ecysone in DNA puff forming sites. Ecdysone injection experiments revealed that the hormone induces DNA puffs formation, DNA amplification and transcription in sciaridae salivary glands (Basso et al., 2002; Foulk et al., 2006).

Experiments in transgenesis have confirmed that the response of DNA puff genes to the hormone is a late response and have shown that the early gene products participate in the regulation of DNA puffs genes (Basso et al., 2002, 2006; Bienz-Tadmor et al., 1991). It will be interesting to learn whether these *trans*-activating factors bind directly or indirectly to the *BhC4-1* promoter, and to further investigate the conservation of the early gene products in sciarids and their role in DNA puff genes regulation. It is worthy of note that the early genes do not regulate either the tissue pattern or the developmental time of the DNA puff gene expression. Furthermore, previous studies from our group have indicated that the *Drosophila* salivary gland transcription factor, Forkhead, does not participate in the regulation of *BhC4-1* gene in transgenesis. In this context, further characterization of DNA puff genes regulatory mechanisms in transgenic *Drosophila* will contribute to the discovery of additional tissue specific transcription factors.

The exciting results demonstrating that the EcR is localized at DNA puff forming sites during DNA puff expansion both in *T. pubescens* and *B. hygida* (Candido-Silva et al., 2008; Stocker et al., 1997), together with EMSA experiments demonstrating that the EcR binds the II/9A *locus* of *S. coprophila* (Foulk et al., 2006) extend the characterization of the role of the EcR in sciarids. One aspect that merits further investigation is the precise role of the EcR. Is the EcR in scarids a transcription factor, an amplification factor or both? The identification of factors that interact with the sciarid EcR during the amplification process, coupled with functional assays employing RNAi, could unravel the mechanism that control DNA amplification during larval development. Furthermore, the recent cloning of the EcR and usp orthologues, both in *B hygida* (Valente V and de Almeida JC, unpublished results, 1999 and Candido-Silva et al., 2008) and *S. coprophila* (Foulk et al., 2006), provides the necessary tools to determine the role of both the receptor and the hormone in DNA puff genes transcription using cell culture based assays. In this context, the study of sciarid DNA puffs will further the knowledge of the roles exerted by the EcR receptor and its ligand, ecdysone.

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