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Differential DNA Replication in Insects

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Insect chromosomes provide wonderful model systems to unravel the mysteries of DNA replication. Data from studies of conventional replication of insect chromosomes are compatible with a mode of semidiscontinuous replication by a set of enzymes similar to those in mammals. Normally, replication initiates just once per S phase in each replicon. During various stages of insect development, however, origins of DNA replication (ORI) at certain loci become the targets of complex developmental signals that lead to their selective activation or repression. As a result, insect chromosomes exhibit several molecular phenomena such as DNA amplification and underrepresentation that are in direct violation of the "rule of DNA constancy."

We believe that these phenomena may yield insights into more general principles for DNA replication control; understanding how a given ORI is driven to fire more than once (or not at all) during S phase may define the molecular mechanisms that direct an ORI to fire once and only once per cell cycle during conventional replication. In this chapter, we focus on the use of differential DNA replication in insect chromosomes as a model system to identify ORIs and explore the regulation of their activation.

Many insect tissues become polyploid or polytene, and certain cell-cycle controls are overridden during these processes. The chapter by Carminati and Orr-Weaver (this volume) describes studies on *Drosophila* mutants that have provided insights into controls for progression through the cell cycle.

CONVENTIONAL DNA REPLICATION IN INSECTS

Replicons with Bidirectional Replication

Much of our understanding of the mechanism for bidirectional replication is based on observations in *Drosophila* embryos, where the rapid

DNA Replication in Eukaryotic Cells

successive rounds of DNA replication and cell division made it possible to visualize many replication bubbles, or "eye-forms" (Wolstenholme 1973; Kriegstein and Hogness 1974; Zakian 1976). The appearance of a single-stranded gap on one side of each replication fork, which can disappear due to branch migration and result in a single-stranded tail of 200 nucleotides on the other side of the fork, suggested that the gap was where the next Okazaki fragment would be placed and supported the concept of semi-discontinuous replication. Okazaki fragments, primed by an octaribonucleotide, have indeed been isolated from *Drosophila* (Blumenthal and Clark 1977; Kitani et al. 1984); it is postulated that a 61-nucleotide replication intermediate gives rise to 125-nucleotide and 240-nucleotide molecules.

The early *Drosophila* embryo has been the target of much biochemical study on the enzymology of DNA replication, since it has a molecular stockpile of enzymes and protein cofactors needed to ensure rapid rounds of chromosome duplication. Recent results of this work are summarized in Table 1, which updates a similar table published in a previous review (Spradling and Orr-Weaver 1987). Since SV40 ORI-dependent replication can be carried out by a *Drosophila* extract supplemented with T antigen (Kamakaka et al. 1994), it appears that the *Drosophila* replication machinery is highly similar to that of other well-studied vertebrate systems (see appropriate chapters in this book).

We hypothesize that even though most of the proteins have been isolated from embryos, it may be that these same factors are used in all subsequent replication events (normal replication during development, replication during polytenization, or DNA amplification). The differential DNA synthesis characteristic for some of these processes could be due to control of ORI activity by factors that do not belong to the basic subset listed in Table 1.

DNA fiber autoradiography data from *Drosophila* have shown that there are many replicons in the genome, which can be visualized to carry out DNA synthesis in a bidirectional manner from an ORI located in the center of each replicon (Steinemann 1981a,b). In *Drosophila* brain cells and tissue-culture cells, replication forks move at 0.35–1.0 μm/min (Blumenthal et al. 1974; Ananiev et al. 1977; Steinemann 1981a), which is up to ten times faster than the rate in polytene chromosomes (Cordeiro and Meneghini 1973; Steinemann 1981b; Lakhotia and Sinha 1983).

Nucleotide-level mapping of a nuclear ORI of bidirectional replication (i.e., the transition point between continuous and discontinuous DNA synthesis) has not been achieved yet in any metazoan, with the exception of some metazoan viruses (Hay and DePamphilis 1982;

Table 1 DNA replication enzymes and protein cofactors isolated from early *Drosophila* embryos

Protein	Subunit (function)	Gene cloned?	References
DNA polymerase-δ	138 (catalytic),	no	Aoyagi et al. (1994)
	or 120 (catalytic)	no	Chiang et al. (1993)
	47	no	Aoyagi et al. (1994)
PCNA	36 (pol-δ cofactor)	yes	Yamaguchi et al. (1990) Ng et al. (1990)
DNA polymerase-α	50 (primase)	yes	Bakkenist and Cotterill (1994)
	60	no	Lehman and Kaguni (1989)
	170 (catalytic)	yes	Hirose et al. (1991)
	or 180 (catalytic)	yes	Melov et al. (1992)
	73	yes	Cotterill et al. (1992)
DNA ligase 1	125 (replication)	no	Rabin et al. (1986) Takahashi and Senshu (1987)
DNA ligase 2	70 (repair?)	no	Takahashi and Tomizawa (1990)
Topoisomerase 1	135	yes	Hsieh et al. (1992)
Topoisomerase 2	170	yes	Wyckoff et al. (1989)
RNase H	2 x 49, 2 x 39	no	DiFrancesco and Lehman (1985)
RP-A	66, 31, 8	no	Marton et al. (1994)
	or 70, 30, 8	no	Mitsis et al. (1993)

Weights are given in kilodaltons, and two references are listed when there is a discrepancy in the literature on the apparent molecular weight of the protein. Proteins associated with types of DNA metabolism other than replication (e.g., repair) are not listed.

Hendrickson et al. 1987). ORIs used in DNA amplification have, however, been mapped to zones of one or several kilobases in insect chromosomes (Delidakis and Kafatos 1989; Heck and Spradling 1990; Liang et al. 1993; Liang and Gerbi 1994), as described in more detail below.

Regulation of Initiation during S Phase

DNA fiber autoradiography of samples from *Drosophila* (Blumenthal et al. 1974; Steinemann 1981b) revealed that the spacing between ORIs varies during development (the mean replicon length in *Drosophila* em-

bryos is 7.9–10 kb; see Blumenthal et al. 1974; McKnight and Miller 1977). This suggested that many ORIs may be activated during early development resulting in a short S phase and allowing rapid cell division. Later, as tissues differentiate and the length of the cell cycle increases, many of these ORIs would become inactive, and the stretch of DNA surrounding such silent ORIs would be replicated by forks that were initiated at adjacent loci. Consequently, the replicon length would be increased.

This hypothesis presumes that there are specific sequences used as ORIs. There is little information on this point. Alternatively, if there is lack of sequence specificity for initiation, other factors such as chromatin structure or the availability of replication enzymes could determine the spacing between ORIs and hence the length of the replicon. However, even if there is lack of sequence specificity to define the position of initiation of conventional chromosomal replication, controls must still exist to prevent re-initiation in the same S phase, since nested replication forks have not been observed in electron micrographs of replicating *Drosophila* embryonic DNA (Blumenthal et al. 1974; Kriegstein and Hogness 1974).

Cytological studies of [^{3}H]thymidine uptake in giant polytene chromosomes of the larval salivary glands of dipteran insects indicated that replicons can vary in the time during S phase when they are actively synthesizing DNA (Keyl and Pelling 1963; Plaut 1963; Gabrusewycz-Garcia 1964; Plaut et al. 1966; Hägele 1970, 1973; Arcos-Terán 1972; Steinemann 1981b). Replicons in heterochromatin are active later in S than are those in euchromatin, and diploid cells also abide by this conclusion (Steinemann 1980).

Deviations from the Rule of DNA Constancy

Even though replicons can initiate DNA replication at different times, generally, by the completion of S phase all of the genomic DNA will have been replicated once and only once. This satisfies the rule of DNA constancy, which states that all cells in an organism have the same DNA content per genome equivalent (Boivin et al. 1948; Swift 1950a,b; Mirsky and Ris 1951). Insect chromosomes, however, present an exception to this rule. As described below, centromeric heterochromatin as well as the ribosomal DNA (rDNA) locus are both underrepresented in *Drosophila* polytene chromosomes; on the other hand, extra DNA is synthesized during amplification of *Drosophila* follicle cell chorion genes and *Sciara* polytene chromosome DNA puffs.

DNA sequence underrepresentation and intrachromosomal DNA amplification have both been ascribed to differential replication. It has been proposed (Laird 1973; Laird et al. 1974) that continuity of DNA through the chromatid is made possible by nested replication forks (Fig. 1); this "onion skin" model was experimentally confirmed in the case of *Drosophila* chorion genes by electron microscopy (Osheim and Miller 1983; Osheim et al. 1988). On the basis of cytological quantitation it was also suggested that nested replication forks might occur at each polytene band/interband boundary (Sorsa 1974; Laird 1980), but except for a few constrictions, this is not supported by molecular hybridization data (Lifschytz 1983; Spierer and Spierer 1984; Lamb and Laird 1987).

Failure to find replication forks at the junction of euchromatin and underrepresented heterochromatic sequences in a mini-polytene chromosome has led to an alternate model according to which the underrepresented sequences are physically excised during polytenization (Karpen and Spradling 1990; Glaser et al. 1992). In fact, although most of the literature presumes that underrepresented sequences have been underreplicated, there are few data to rule out the alternate model of excision.

UNDERREPRESENTED SEQUENCES IN DIPTERAN CHROMOSOMES

In dipteran salivary glands, and to a lesser extent in most other larval tissues (Ashburner 1970), chromosomes undergo polytenization—rounds of endoduplication without intervening cell division (Swift 1962; Rasch 1970b)—with the sister chromatids remaining synapsed together and not separating as they do in polyploid nuclei. Parts of the genome, such as the heterochromatic Y chromosome that is not represented in *Drosophila* salivary gland polytene chromosomes (Painter 1933; Lindsley and Grell 1967; Holmquist 1975; Zhang and Spradling 1995), can escape the endo-

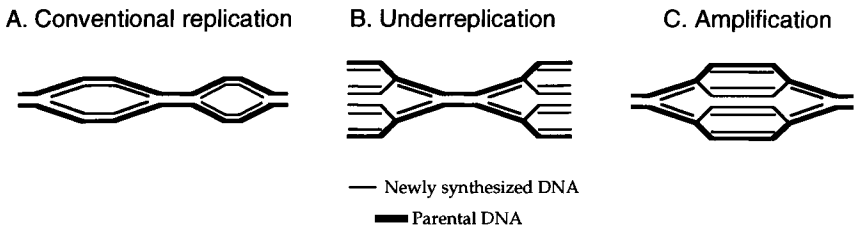

Figure 1 Models of two adjacent eye-forms for conventional replication (*A*), nested replication forks for underreplication (*B*) and DNA amplification (*C*), according to the "onion skin" model (Laird 1973; Laird et al. 1974).

duplication process. Not only whole chromosomes, but also individual loci can be subject to regulation of their representation in the genome, as exemplified by satellite DNAs and rDNA as described below.

Centromeric Satellite DNA

Heitz (1934) was the first to propose that during polytenization the amount of centromeric heterochromatin is reduced relative to euchromatin. For simple sequence satellite DNA, found in the heterochromatin of *Drosophila* polytene chromosomes (Gall and Atherton 1974; Peacock et al. 1974; Steinemann 1976), this was verified by comparing polytene and diploid cells in three ways: (1) by microspectrophotometry (Berendes and Keyl 1967; Mulder et al. 1968; Rudkin 1969), (2) by in situ hybridization (Gall et al. 1971; Peacock et al. 1974), and (3) by analytical ultracentrifugation in CsCl gradients (Dickson et al. 1971; Gall et al. 1971; Cordeiro et al. 1975). In contrast, the satellite DNA of Chironomids remains fully represented in the genome during polytenization (Walter 1973; Steinemann 1978).

Underrepresentation of satellite DNA relative to the rest of the genome is not limited to salivary gland polytene chromosomes but can occur in other tissues as well, such as nurse cells and follicle cells (Hammond and Laird 1985). When comparing DNA from *Drosophila virilis* diploid cells (embryos or adult head and brain) to adult tissues that are often polyploid, it was observed that the satellite DNAs were underrepresented to different levels in the latter (Blumenfeld and Forrest 1972; Schweber 1974; Endow and Gall 1975). Underrepresentation of satellite DNA also occurs in some other insects (Kunz and Eckhardt 1974; Redfern 1981), but in some cases satellite DNA is fully present in ovarian nurse cells (Gall et al. 1969; Zacharias 1979; Nazimiec and Beckingham 1986). Interestingly, satellite DNA can also be overrepresented relative to the rest of the genome, as is the case for pupal ovaries of *D. virilis* (Endow and Gall 1975), although with time after eclosion of the adult fly it becomes underrepresented (Endow and Gall 1975; Renkawitz-Pohl and Kunz 1975). In general, either underreplication or excision can be used equally well to account for all instances of satellite DNA underrepresentation in polytene or polyploid cells.

Ribosomal DNA

rDNA Underrepresentation during Polytenization

There is some underrepresentation of rDNA relative to the rest of the genome during polytenization of salivary gland chromosomes in *D. hydei*

(Hennig and Meer 1971) and *D. melanogaster* (Spear and Gall 1973; Spear 1974; Szabo et al. 1977), where it appears to lag behind by an average of three polytene rounds. This is not as severe as simple sequence satellite DNA, which is hardly represented at all in polytene cells. rDNA underrepresentation can also occur in polyploid tissues, such as the ovary of adult *Drosophila*, resulting in almost half its level in diploid cells (Endow and Gall 1975; Renkawitz and Kunz 1975). In contrast, there is no underrepresentation of rDNA in salivary gland polytene chromosomes of *Chironomus tentans*, which lacks heterochromatin (Hollenberg 1976); in Sciarid flies the conclusions are less clear (Gerbi 1971; Gambarini and Meneghini 1972). Moreover, in *D. hydei* nonheterochromatic moderately repetitive sequences like the 5S RNA genes appear to replicate with the rest of the genome (Renkawitz-Pohl 1978).

rDNA is less severely underrepresented in nurse cells that synthesize all the rRNA of the oocyte (Renkawitz and Kunz 1975; Beckingham and Thompson 1982; Hammond and Laird 1985), suggesting a functional selection for its presence. In addition, those rDNA repeat units that contain insertions and are not transcribed (for review, see Beckingham 1982) are underrepresented even more in polytene and polyploid cells than the intron$^-$-functional rDNA units (Endow and Glover 1979; Beckingham and Thompson 1982). Once again, the downward adjustment in copy number could be the result of either failure to replicate or sequence excision.

rDNA Amplification: Compensation and Magnification

Under certain circumstances rDNA loci can also undergo amplification. Specifically, *Drosophila* that are XO rather than XX and have one instead of two nucleolus organizer regions contain the same amount of rDNA in salivary glands as do XX individuals (Spear and Gall 1973). This somatic compensation may be due to regulation of the polyteny level of the nucleolus organizer region by overreplication (Spear and Gall 1973; Endow and Glover 1979; Endow 1980, 1982) and could also account for the relative rDNA increase in adult XO flies (Tartof 1971, 1973).

rDNA levels can also change in the germ line. The standard constitution of the nucleolus organizer region on either the X or the Y chromosome of *Drosophila* is about 200 tandemly repeated rDNA copies. The *bobbed* phenotype reflects a deficiency in the number of rDNA copies (Ritossa et al. 1966). When male flies have a deficiency in the number of rDNA copies on both the X (X^{bb}) and Y chromosomes (Y^{bb-}), they

transmit X chromosomes with more rDNA than was originally carried, and after a few generations the standard level of rDNA in the nucleolus organizer region is restored. This process was named "magnification" by Ritossa (1968). Both the intron$^+$ and intron$^-$ rDNA copies are equally amplified (de Cicco and Glover 1983). Magnification can also occur in female flies deficient in rDNA provided that they carry the tip of the long arm of the Y chromosome (Komma and Endow 1986, 1987).

rDNA magnification has been hypothesized to result from replication and subsequent reintegration of extrachromosomal rDNA rings that were derived from the chromosomal nucleolus organizer region (Ritossa 1972; Locker and Prud'homme 1973; Ritossa et al. 1974; Locker 1976). However, Tartof (1974a,b) proposed an alternate hypothesis of unequal sister chromatid exchange, and recent data support this model (Endow et al. 1984; Endow and Atwood 1988; Komma and Atwood 1994). The reciprocal product of unequal exchange for magnification (bb^m) is the reduced genotype (bb^r) of a still greater deficiency in rDNA copies; the reduced genotype is rarely observed, perhaps due to selective pressure against it.

INTRACHROMOSOMAL DNA AMPLIFICATION IN DIPTERA

The examples described below of intrachromosomal DNA amplification of the *Drosophila* chorion loci and *Sciara* DNA puffs both occur as a normal, developmentally regulated event in terminally differentiated tissue. In fact, such significant deviations from the rule of DNA constancy may be a luxury enjoyed only by systems where cells are destined to degenerate shortly thereafter (the follicle cells degenerate after stage 14 and the larval salivary glands are destroyed in the subsequent pupal stage) and will not have to carry the burden of extra DNA in their genome for a long time after amplification.

It is conceivable that intrachromosomal gene amplification could also occur in the germ line, in which case it would be passed on to the resulting offspring as a stably maintained increase in that gene. One example of this was just described for rDNA magnification, although this appears to result from recombination rather than DNA overreplication. Another example is found when comparing 30 polytene bands between *Chironomus thummi thummi* and *Chironomus thummi piger* (Keyl 1965a,b). The *piger* line shows increased DNA relative to the *thummi* line in a geometric series, suggesting that extra rounds of replication occurred in the germ-line replicons of the *piger* ancestor. This phenomenon

could be related perhaps to the finding that the tandemly repeated Cla elements are dispersed throughout all chromosomes of *C. thummi thummi* but are limited to the centromeric regions of *C. thummi piger* (Hankeln et al. 1994).

These exceptions are all examples of *intra*chromosomal DNA amplification of a locus relative to its usual representation in the genome. *Extra*chromosomal gene amplification can also occur (e.g., rDNA in amphibian oocytes; see Brown and Dawid 1968; Gall 1968), but a return to the standard composition of the genome is easily accomplished by an apparent failure to replicate and dilution out of the extra rDNA copies during subsequent cell divisions of the early embryo.

DNA Amplification in the Chorion Loci of *Drosophila*

During the last 16 hours of oogenesis in *D. melanogaster*, there is amplification in the ovarian follicle cells of the two clusters of chorion genes, on the X and third chromosome. The amplified genes provide extra template for the ensuing extensive production of eggshell proteins. The chorion cluster on the X chromosome amplifies 16- to 20-fold, and the cluster on the third chromosome amplifies 60- to 80-fold (Spradling and Mahowald 1980; Spradling 1981). Re-initiation is frequent (2.5-hr doubling time) and the replication forks elongate slowly (50–100 bp/min) (Spradling and Leys 1988). For both chorion clusters there is a gradient of amplification spanning almost 100 kb (Spradling 1981), suggesting that the peak of amplification in the center of the gradient may correspond to a repeatedly firing origin.

Delidakis and Kafatos (1989) and Heck and Spradling (1990) have mapped the ORI for amplification of the chorion locus on the third chromosome by two-dimensional gels (see Fig. 2 for schematic map). A major initiation zone of 7.7 kb and a minor initiation zone of at least 4.2 kb were revealed. Furthermore, it was speculated that a 1-kb region within the 7.7-kb zone may be the major ORI.

A search has begun for *trans*-acting factors that regulate chorion gene amplification. Seven female-sterile mutants with defects in chorion gene amplification have been found (Komitopoulou et al. 1986; Orr et al. 1984; Kelley and Spradling 1986; Snyder et al. 1986; Underwood et al. 1990), but clones encoding these factors have not yet been isolated, and their identity remains unknown.

Some of these unidentified *trans*-acting factors presumably bind to the *cis*-regulatory sequences important for *Drosophila* chorion gene

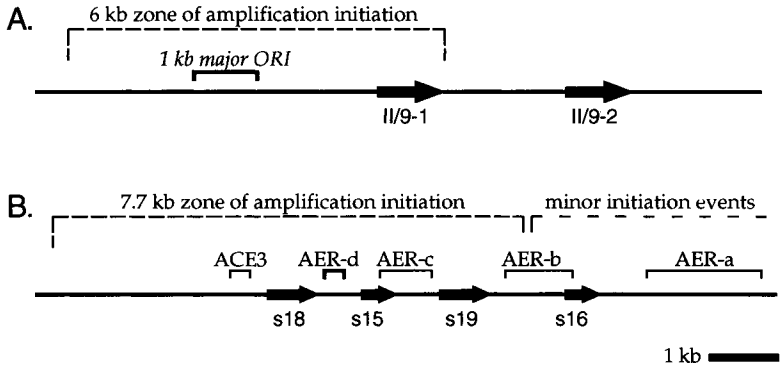

Figure 2 Schematic maps of (*A*) amplified loci for *Sciara* DNA puff II/9B (Liang et al. 1993; Liang and Gerbi 1994) and (*B*) *Drosophila* chorion genes (Delidakis and Kafatos 1989; Heck and Spradling 1990). Major origin activity is presumed to lie in AER-d (also called ORI-β), denoted by a darkened bracket. See text for explanations of abbreviations.

amplification. One such sequence, ACE3 (*a*mplification *c*ontrol *e*lement on the third chromosome) has been delimited by P-element transformation to a 320-bp region upstream of gene s18 and was separate from the transcription control region (Orr-Weaver et al. 1989). ACE3 contains an evolutionarily conserved 71-bp motif, although additional nonconserved sequences are also needed for its function (Swimmer et al. 1990). Similarly, ACE1 that regulates chorion gene amplification on the X chromosome has an essential 467-bp region with repeated sequences necessary for amplification but not for transcription (Spradling et al. 1987). Transposons bearing 1, 9, or 18 copies of 400 bp of ACE3 in a tandem array are sufficient to drive amplification, but only to low levels (Carminati et al. 1992). It remains unclear whether ACE3 contains an ORI or whether it stimulates replication from an adjacent ORI.

In addition to ACE3, there are four *a*mplification *e*nhancing *r*egions (AER a–d) whose deletion reduces but does not eliminate amplification in transformant lines (Delidakis and Kafatos 1987, 1989). Some transposons containing the AERs but lacking ACE3 can amplify to low levels (Swimmer et al. 1989). Data from such an approach need to be interpreted with caution due to the sensitivity of chorion gene amplification to position effects (these become more severe as the size of the DNA fragment to be tested decreases; de Cicco and Spradling 1984). The precise functional roles and interrelationships of ACE3 and the AERs remain to be elucidated.

DNA Amplification in Sciarid DNA Puffs

Poulson and Metz (1938) were the first to note the large DNA puffs that appear in salivary gland polytene chromosomes of sciarid flies by the end of the fourth instar. Unlike the RNA puffs of *Drosophila* or Balbiani rings of *Chironomus* that exhibit intense RNA transcription without gene amplification (Rudkin 1955; Hägele 1970; Rasch 1970b), the DNA puffs were hypothesized to contain "extra" DNA on the basis of increased Feulgen staining (Breuer and Pavan 1955) and [^{3}H]thymidine uptake (Ficq and Pavan 1957); microspectrophotometry provided definitive proof (Rudkin and Corlette 1957; Swift 1962; Crouse and Keyl 1968; Rasch 1970a).

There are nine major and nine minor DNA puffs in the giant salivary gland chromosomes of the fungus fly, *Sciara coprophila* (Gabrusewycz-Garcia 1964, 1971). We chose puff 9A on chromosome II (II/9A), one of the largest and among the first to appear, for our molecular studies (see schematic map in Fig. 2). There are two transcription units, II/9-1 and II/9-2; these are 85% similar to one another in sequence and encode a putative α-helical coiled-coil protein preceded by a signal sequence for secretion (DiBartolomeis and Gerbi 1989). It has been deduced that the DNA puffs of *Rhynchosciara* encode specific polypeptides to build the pupal cocoon (Winter et al. 1977a,b; de Toledo and Lara 1978; Ferreira and Amabis 1983; Laicine et al. 1984). Presumably, DNA puff amplification occurs to accommodate the need for massive amounts of proteins that form the pupal case.

DNA puff amplification is superimposed on the last chromosomal endoduplication (Crouse and Keyl 1968; Rasch 1970a), and there are about four extra rounds of replication to result in 16- to 22-fold more DNA (Glover et al. 1982; Paçó-Larson et al. 1992; Wu et al. 1993). The extra DNA appears to remain at the DNA puff loci even in very late larval stages that were examined by microspectrophotometry (Crouse and Keyl 1968; Rasch 1970a). DNA amplification precedes maximum puffing; morphological puffing is correlated with an increase in the amount of mRNA that was presumably transcribed off the amplified DNA (Paçó-Larson et al. 1992; Wu et al. 1993). Thus, transcription occurs after amplification has reached its final level in all cases studied except for the C8 DNA puff of *Rhynchosciara* (Santelli et al. 1991; Wu et al. 1993). As might be deduced from the different timing of amplification and puffing during development, inhibition of DNA synthesis by hydroxyurea does not inhibit puff formation (Sauaia et al. 1971).

We have mapped the origin of amplification in DNA puff II/9A by two different two-dimensional gel methods (Liang et al. 1993) and by a

three-dimensional gel method (Liang and Gerbi 1994). Initiation is confined to a 6-kb zone, with the majority of initiation events occurring in a 1-kb region that is 2 kb upstream of gene II/9-1. Replication forks move outward in a bidirectional manner from the amplification ORI (Liang et al. 1993), consistent with, but not proving, an onion-skin model of DNA amplification. It is not yet clear what the boundaries of the amplification gradient are in *Sciara*; however, preliminary data from DNA puff C4 of *Bradysia hygida* suggest that the DNA amplification gradient may be considerably shorter than the 100 kb found in the *Drosophila* chorion loci (Coelho et al. 1993; Monesi et al. 1995).

It would appear from three-dimensional gels that only one and not several clustered initiations occur in the ORI zone of a single DNA molecule (Liang and Gerbi 1994). Preliminary data suggest that the same region is used for initiation of conventional DNA replication earlier in development, as for DNA puff amplification (C. Liang; F.D. Urnov; S.A. Gerbi; all unpubl.).

In a manner similar to the studies on the chorion loci, the search has begun for putative *trans*-acting factors that may be responsible for the activation of the DNA puff amplification. These data are discussed in the following section.

FUTURE DIRECTIONS AND PERSPECTIVES

Our attempts to understand how regulation of replication is achieved such that each replicon initiates once and only once per cell cycle may be illuminated by an examination of exceptions to the rule from insect systems. For instance, if underreplication is the mechanism for underrepresentation, then one insight that arises from the observations listed is that regulation can be executed at the level of the individual replicon. Thus, satellite DNAs could underreplicate to different levels from one another in the same nucleus. Similarly, rDNA units could vary in their replication level depending on whether they carry an intervening sequence or not, and in the ovary, satellite DNA is underrepresented and rDNA amplified in the same cell. It would thus appear that merely placing a given replicon in a heterochromatic domain may be important but not sufficient to inhibit its replication, and some additional regulatory mechanisms reside within each replicon (e.g., rDNA and satellite DNA).

How are the boundaries for differential replication established? In underreplication, if an ORI does not fire, why is the DNA of that replicon not replicated by forks traveling into it from adjacent replicons? Are there discrete termination sites that prevent forks from moving into this

region, or is this regulated by boundaries set up perhaps by chromatin architecture? Similarly, are there boundaries that prevent the extra rounds of replication in amplified DNA puffs from sweeping outward into adjacent chromosomal regions? It is known that re-initiation at the DNA puff II/9A ORI stops by late larval life (Wu et al. 1993), but whether the replication forks continue to move outward until the salivary gland is histolyzed during pupation has not been studied.

It would appear that there is selection for the number of DNA templates needed for transcription in a given cell type. For instance, although the rDNA level may be magnified to even a higher level than normal, ultimately the rDNA copy number is corrected to the normal level (de Cicco and Glover 1983). In another example, the nonfunctional intron$^+$ rDNA copies are less prevalent than intron$^-$ rDNA copies in polytene and polyploid cells, although it is not yet proven whether this is due to underreplication. Finally, extra templates are provided by overreplication of the *Drosophila* chorion loci and sciarid DNA puffs as a result of selective pressure to produce more template for intense transcription from these loci.

Is the ORI a specific sequence, and how is its activity regulated? Specifically, how do ORI silencing mechanisms compare between instances of underreplication and developmental ORI control? Is such silencing an active process requiring an inhibitor for ORI function, or is it simply the lack of an activator for that particular ORI? Similarly, for intrachromosomal amplification, how are the ORIs regulated such that they fire more than once relative to replication of the rest of the genome? Does this process require an activator specific for that ORI, or does it reflect repression or absence of an inhibitor that normally prevents an ORI from firing more than once?

An answer to these questions necessitates the identification of *trans*-acting factors involved, and preliminary data for *Sciara* DNA puffs may shed light on this problem. Specifically, as was deduced in earlier cytological studies (Crouse 1968; Stocker and Pavan 1974; Berendes and Lara 1975; Fresquez 1979; Ferreira and Amabis 1980; Amabis and Amabis 1984a,b; Stocker et al. 1984; Dessen and Perondini 1985; Alvarenga et al. 1991) and shown by quantitative Southern blots (M. Batra; D. Alam; C. Liang; H.S. Smith; S.A. Gerbi; all unpubl.), DNA puff amplification can be prematurely induced in cultured salivary glands from young larvae by the steroid hormone, ecdysone, which also regulates transcription of DNA puff genes (for references, see above and Bienz-Tadmor et al. 1991). Several examples are known in viral systems where the same protein regulates both replication and transcription. Is the

ecdysone receptor acting in a similar way in DNA puffs? Models on how this might be accomplished have been proposed (Lara et al. 1991; Gerbi et al. 1993) and await results from future experiments for their verification.

A full understanding of the target of regulatory *trans*-acting factors requires a better characterization of the ORI itself. The endogenous pattern of DNA replication is not maintained when *Drosophila* polytene nuclei replicate in *Xenopus* egg and oocyte extracts (Sleeman et al. 1992). *D. melanogaster* DNA fragments that function as autonomously replicating sequences (ARS) in yeast fail to do so in *Drosophila* embryos (Roth 1991), and plasmids containing *D. melanogaster* DNA appear to replicate without sequence specificity for initiation in cultured *Drosophila* cells (J.G. Smith and M.P. Calos, in prep.). Therefore, the ARS assay that has been so powerful in yeast does not seem fruitful for identifying critical regions for ORI function in insect DNA. However, data from these experiments should not be taken to imply that there is no sequence specificity for the ORI, since two- and three-dimensional gel analyses on DNA from the *Drosophila* chorion loci and *Sciara* DNA puffs demonstrate localizaton of the ORI for amplification to defined regions. Perhaps P-element transformation where the DNA to be tested is in a chromosomal context rather than on an extrachromosomal plasmid may be the best way to further dissect the properties of these ORIs. Future studies are needed to determine what additional molecular elements are necessary to produce amplification or underreplication, beyond the normal controls that a cell exerts over an ORI for conventional replication.

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