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# 6 Comparative Aspects of Chromosome Replication in *Drosophila* and Mammals

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The double helical model for DNA structure provides a self-replicating property to the molecule. Essentially, DNA replication requires polymerization of complementary chain of nucleotides on a template. Fulfilment of this requirement, however, involves an elaborate enzymatic machinery even for the simplest of organisms where the genetic material could be a naked DNA molecule, only a few kilobases long (Kornberg, 1980). Although the basic process has been conserved through the evolutionary time scale, the increasing complexity of the genome has correspondingly resulted in a more intricate mechanism of replication in higher organisms. The genetic material in eukaryotes is not only compartmentalized in the nucleus, but is also distributed in different linkage groups and is complexed with a variety of proteins, some permanent others transient (see Sen and Kuo, this volume). The genetic material thus exists as chromatin and becomes visible as discrete individual units, the chromosomes, in certain stages of the cell reproduction cycle. Unlike in prokaryotes, cell division does not immediately follow replication nor does replication necessarily follow division. Two periods of growth respectively span cell division and DNA replication ($G_1$), and vice-versa ($G_2$). This, however, leaves a well defined period in the cell division cycle, the S-phase, in which replication is effected.

Individual chromosomes comprise large amounts of DNA and while their number and sizes vary within and between taxa, an average-sized chromosome has several-fold more DNA than in *E. coli*. Yet the ability of a cell to replicate so much DNA within a few hours indicates that replication proceeds in different chromosomes and in different regions of the same chromosome at the same time. However, as will be discussed later, not all the DNA is replicated simultaneously and segments of DNA are programmed to replicate at different but generally predetermined periods within the S-phase. Thus the increase in the amount of DNA, its compaction into chromatin and confinement of replication to a limited period in the cell cycle have made the process of replication more elaborate in eukaryotes than in prokaryotes.

Studies on chromosome replication were in-

itially handicapped by the fact that chromatin is generally cytologically invisible at the time of replication (S-phase). The advent of the cellular autoradiography technique and the commercial availability of radiolabelled nucleic acid precursors brought in their wake a volume of data on chromosomal replication. In the simplest form of technique, mitotically cycling cells are grown in a medium containing $^3$H-thymidine for desired periods so that the cells that are in S-phase take up the label at the sites of ongoing DNA synthesis and these cells are then allowed to progress to metaphase before the cells are harvested for cell autoradiographic studies. The slides containing chromosome preparations are coated with a fine-grained photographic emulsion and exposed in darkness for a certain period of time before photographic development. $^3$H-thymidine, wherever incorporated during the S-phase, is revealed on the chromosomes as dark silver grains. Extremely close proximity of chromosomes with the photographic emulsion ensures a reasonably accurate representation of the sites replicating at the time of $^3$H-thymidine incorporation. There are cell types where chromosomes are visible during their synthetic phase itself, e.g. polytene cells in diptera and certain other organisms. Obviously, in these cases, $^3$H-thymidine incorporation is not required to be chased up to metaphase, instead it can be visualized directly. In the case of mitotically cycling cells also, a comparable condition can be experimentally simulated by premature chromosome condensation of interphase chromatin by fusion with mitotic cells (Sperling and Rao, 1974).

A major advance in the study of chromosome replication, especially in mammalian cells, was the development of a variety of chromosome banding (C, G, Q, R-bands, etc.) techniques and the use of 5-bromodeoxyuridine as the label for newly synthesized DNA. While the various banding techniques allowed a better resolution of structure of mitotic chromosomes which otherwise looked monotonously uniform, the bromodeoxyuridine label bypassed the requirement of the autoradiographic step since special methods of fluorescent or Giemsa staining directly allowed a precise visualization of sites of incorporation of the label with a much higher degree of resolution than that obtained with the autoradiographic technique (see Zakharov and Egolina, 1972; Latt, 1973).

The present chapter describes some aspects of replication at chromosomal level, especially features that regulate the S-phase and the temporal order of chromosomal replication in relation to chromatin activity and cell differentiation. Since our own areas of research are confined to mammals and *Drosophila*, the discussion will mainly be confined to these two systems with an attempt to bring out similarities and differences in their strategies for chromosomal replication.

## Different Strategies of Chromosomal Replication in Mammals and *Drosophila*

In a majority of organisms, including mammals, cell proliferation is the main source of growth and differentiation. In a typical cell proliferation cycle, the products of a mitotic division undergo a variable period of cell growth (the $G_1$-period) preparatory to the following S- or the DNA replication phase. The $G_2$-period which follows the S-phase prepares the cell for entry into mitosis which typically involves chromosome condensation, an orderly separation of sister chromatids to two poles, reformation of nuclear envelopes around the two daughter nuclei and finally cytokinesis to complete the generation of two daughter cells (Prescott, 1976). In several groups of eukaryotes, however, certain cell types short circuit the regular mitotic cell cycle in various ways to achieve somatic growth and differentiation (see Rudkin, 1972, Nägl, 1978).

For example, in a number of tissues of *Drosophila*, the post-S-phase cells, instead of entering the cell division cycle, resume a fresh cycle of DNA synthesis following a pause of varying duration. Thus in organisms like *Drosophila*, somatic growth is achieved largely by endoreplication and cell growth following an initial phase of cell multiplication. While the early embryonic and those larval cells that are destined to form adult structures proliferate mitotically, a majority of larval and some of the adult cell types result from a well regulated pattern of endoreplication cycles. It is also notable that in the adult stage of *Drosophila*, DNA synthesis occurs only in gonadal cells.

## General Aspects of Replication and Chromosome Structure in *Drosophila*

*Drosophila* provides examples of mitotic and endoreplication cycles in its different cell types and at different stages of development. The occurrence of endoreplication cycles in somatic cells of *Drosophila* (and other similar organisms) can lead to at least two types of nuclear organizations, viz., the well known polytene types with distinct band-interband organization of chromosomes in interphase nucleus, and the polyneme type (see Rieger *et al.*, 1976 for definitions) in which all the daughter chromatids of different chromosomes remain less tightly synapsed so that the nucleus looks more like a typical interphase nucleus but much larger in size and DNA content. An interesting aspect of endoreplication cycles, *Drosophila* is the occurrence of specific patterns of unequal replication of different DNA sequences on the same chromosome. This under- and/or over-replication raises intriguing possibilities about chromosome structure in these endoreplicating polytene or polyneme cell types.

Among the non-polytene tissues, the brain ganglia of *Drosophila* larvae have received considerable attention for studies on chromosomal structure and replication since this tissue provides the most common source for studies on mitotic chromosomes in *Drosophila*. Although not so commonly appreciated, the brain ganglia of *Drosophila* larvae also contain endoreplicating cells. Evidence for endoreplication in larval brain cells was first provided by Berendes and Keyl (1967) who found by cytophotometric measurements that the DNA content in different interphase cells in brain ganglia of *D. hydei* larvae was not in geometric multiples of the 2C values; rather the hetero- and euchromatin components appeared to endoreplicate to independently varying levels. Occurrence of such independent endoreplication cycles of hetero- and euchromatin regions in different brain cells in *D. nasuta* larvae was also shown by autoradiographic and fluorescence studies by Lakhotia and Kumar (1980). Lakhotia (1982) further raised the possibility that some of these endoreplicating brain cells are triggered to enter mitotic division cycles and since these mitotic cells display typical 2N metaphase figures, the metaphase chromosomes in these cell types could be polynemic as suggested by several cytophotometric studies earlier (Rudkin, 1963; Swift, 1965; Gay *et al.*, 1970). However, another cytophotometric study has contradicted the polynemy concept (van de Flierdt, 1975). Since the patterns of segregation of newly synthesized DNA in daughter chromatids can be followed and since these patterns provide information about the uninemic or polynemic structure of mitotic chromosomes (see Prescott, 1970), a detailed analysis of cell cycle and chromosomal labelling patterns in mitotically dividing cells of *Drosophila* becomes specially important for understanding their metaphase chromosome structure.

Unfortunately, studies of chromosomal replication and cell cycle patterns in these mitotically dividing larval cell types are rather few in

the published literature (Pimpinelli *et al.*, 1976; Wienberg, 1977; Tsuji and Tobari, 1980; Steinemann, 1980) and these, too, have been based on equivocal assumptions. Steinemann (1980), using the conventional $^3$H-thymidine pulse-chase method but scoring from a small metaphase sample, estimated the neuroblast cell cycle in *D. virilis* larvae to be about 23h long (S = 12h; $G_2$ = 7h). However, a basic assumption in Steinemann's (1980) study that the replicating cells in larval brain ganglia constitute an exponentially proliferating population of cells is also not valid in view of the known presence of a significant number of endoreplicating cells in the larval brain (see above). Lakhotia *et al.* (1979) found at least some of the larval brain cells in *D. nasuta* to complete cell cycle within 1–2h. In another more extensive study, Roy (1983) noted that the percentage of labelled metaphases in brain ganglia of *D. nasuta* larvae pulse labelled with $^3$H-thymidine for 10–15 min and chased for 15 min to 48h, remained greater than 50 per cent in nearly all samples and thus the cell cycle parameters could not be estimated conventionally. Study of cell cycle parameters in the brain ganglia of *Drosophila* larvae is interesting but difficult to interpret since this tissue includes both mitotically dividing and endoreplicating cells. If the mitotic cells in larval brain ganglia are indeed polynemic as some of the evidence may suggest, it implies that the metaphase chromosome organization in early cleavage division cells and in larval brain cells differs and that the patterns of chromosomal replication cycles bring about this difference. A difference in the mitotic chromosome organization between embryonic and larval brain cells was also noted in studies on the sensitivity of these chromosomes to exposure to certain DNA-ligands like Hoechst 33258, Distamycin A, etc. (Lakhotia and Roy, 1981, 1983; Dolfini and Razzini, 1983; Dolfini, 1987). Thus the replication cycles in larval brain ganglia continue to raise intriguing questions.

A very striking feature of endoreplication cycles in *Drosophila* and several other organisms is a tissue- and developmental stage-specific regulation of participation of different DNA sequences in endoreplication. Classical observations of Heitz (1934) and numerous other subsequent studies (Rudkin, 1964; Gall *et al.*, 1971; Lakhotia, 1974, 1984; Hammond and Laird, 1985a) have shown that the bulk of pericentric heterochromatin regions does not endoreplicate during polytenization in salivary glands of *Drosophila* larvae. Notwithstanding some recent claims to the contrary (e.g. see Lamb, 1982; Dennhoffer, 1982a, b), it is now well established that in nearly all polytene cell types in *Drosophila*, the pericentric heterochromatin regions, including the entire Y-chromosome in males, remain grossly under-replicated although the extent of under-replication may vary in a narrow range in relation to developmental stage and tissue type (see Lakhotia, 1984; Hammond and Laird, 1985a, b; Zacharias, 1986; Spradling and Orr-Weaver, 1987; P.K. Tiwari and Lakhotia, unpublished).

A number of other specific sequences are also known to be underreplicated in salivary gland polytene nuclei of *Drosophila* larvae. These include ribosomal sequences, histone genes, nearly all intercalary heterochromatin sites, the bithorax locus, etc. (for a detailed discussion and references, see Spradling and Orr-Weaver, 1987). In view of these instances of under-replication and several other considerations, Laird (1980) proposed an interesting model of polytene chromosome organization in *Drosophila* larvae where it was suggested that the level of polyteny varies along the length of a chromosome arm such that the interbands are generally less polytenized than the bands (also see Lakhotia and Sinha, 1983). Although Spierer and Spierer (1984) did not find any difference in the levels of polyteny in interbands and bands in a specific region of the 3rd chromosome, a vari-

able polytenization along the euchromatic arms seems to be a reality (Zhimulev *et al.*, 1982; Lamb and Laird, 1987). This may not, however, be as extensive as proposed by Laird (1980). These instances of under-replication and consequent differences in polyteny levels along the length of a chromosome arm, require the presence of cascades of arrested replication forks on either sides of the under-replication domains.

The patterns of differential replication in other endo replicating cell types of *Drosophila* have been studied less extensively. Endow and Gall (1975) found the relative amounts of satellite sequences in different polytene and non-polytene cell types of *D. virilis* to vary, which suggested a tissue-specific pattern of differential replication of these sequences during endoreplication cycles. The larval brain ganglia have generally been taken as representatives of diploid cell types because they show typical diploid mitotic figures and also because the relative amounts of satellite sequences in this tissue were found to be typical for the species. However, the progression of endoreplication cycles in many of the larval brain cells is well documented cytologically and since the heterochromatin may be under- or over-replicated in different brain cells (Berendes and Keyl, 1967; Lakhotia and Kumar, 1980), it is possible that biochemical estimations of satellite DNA content in this tissue would fail to detect any change due to the averaging effect (Lakhotia, 1982). Grimm and Kunz (1980) and Grimm *et al.* (1984) reported over-replication of rDNA sequences in brain ganglia and/or thoracic muscles in certain specific genotypes of *D. hydei*. Starting with the presumption that the brain tissue is essentially diploid, these authors suggested that this over-replication of rDNA in certain genotypic combinations in 'diploid' cells is distinct from the regulated under-replication, magnification, etc. that occur in other polytene cell types. However, in view of the above, the over-replication of rDNA sequences in brain ganglia of *D. hydei* (Grimm *et al.*, 1984) could also be linked to independent endo-replication cycles of euchromatin and heterochromatin (within which the rDNA sequences are located) in a significant proportion of brain cells.

A different category of differential replication in *Drosophila* cells is the amplification of chorion gene clusters in follicle cells at specific stages of ovarian follicle development. This aspect has been reviewed periodically (for details, see Kafatos *et al.*, 1985a, b; Kalfayan *et al.*, 1985; Spradling and Orr-Weaver, 1987). So far no other instances of gene amplification are known in *Drosophila*, although DNA puffs, which reflect gene amplification in polytene chromosomes, are known in some other dipterans like *Rhynchosciara* (Gabrusewycz-Garcia, 1964; Amabis and Amabis, 1984; Spradling and Orr-Weaver, 1987).

In summary, the control of replication, mitotic vs endoreplication cycles, is a very important aspect of cell differentiation in *Drosophila*. Moreover, in cells that are channellized to enter endoreplication pathways, the *Drosophila* genome can identify specific DNA sequences and regulate their participation in replication such that they show characteristic levels of under- or over-replication. The precise mechanisms that effect this intricate regulation are not known, but as may be expected, a rather large number of gene loci affecting replication patterns of specific cell types and/or DNA sequences have been identified in *D. melanogaster* (reviewed in Spradling and Orr Weaver, 1987). Hormonal and developmental conditions also modulate replication patterns of specific cell types. From this point of view, cell differentiation in a typical mammalian system shows certain very basic differences from that in *Drosophila*. Control of replication, i.e., the number and timing of mitotic division cycles are of course also important in mammalian cells but these replication cycles do not, in general, bring

about such extensive qualitative and quantitative changes in the genomic content of differentiating cells as in *Drosophila* and similar other systems.

## Temporal Sequence of Chromosomal Replication

Initial studies on chromosomal replication in mitotic (Lima-de-Faria, 1959; Taylor, 1960) and polytene cells (Plaut, 1963; Keyl and Pelling, 1963) demonstrated that different chromosomes and regions thereof replicated at different times in the S-phase. One of the earliest (and more or less global) correlations between the time of replication and chromatin structure/organization was the replication of heterochromatin during the late S-phase (Lima-de-Faria and Jaworska, 1968). The literature is replete with observations in both plants and animals that heterochromatin replicates late in the S-phase. The correlation between heterochromatization, genetic inactivity and replication during the late S-phase (Lima-de-Faria and Jaworska, 1968; also see Shah *et al.*, 1974 for review of earlier literature) was regardless of whether the heterochromatin was constitutive or facultative (Brown, 1966) like the inactive or lyonized X-chromosome in somatic cells of female mammals (Lyon, 1961, 1974).

With the advent of metaphase chromosome banding techniques, it was shown by Ganner and Evans (1971) and Sharma and Dhaliwal (1974) in humans and the barking deer, *Muntiacus muntjak*, respectively, that besides heterochromatin, the G-band regions also replicated during the later half of the S-phase. The use of BrdU-Giemsa staining technique has since provided much more detailed and better resolved information on the temporal and regional order of replication in individual mitotic chromosomes in mammalian cells (Stubblefield, 1975; Dutrillaux *et al.*, 1976; Das and Savage, 1978; Popescue and Dipaolo, 1979; Camargo and Cervenka, 1982; Dubey, 1985).

A major aspect of the large number of autoradiographic studies on polytene chromosome replication was to ascertain the temporal order of replication of different chromosomal regions during a given polytene S-period. However, the absence of real time marker in these endoreplication cycles and a varying degree of asynchrony in different nuclei have been limiting factors which necessitated application of other strategies for analysis (see Rudkin, 1972 for review). It is now well established that during the later polytene replication cycles, a given S-phase is initiated by replication at disperse regions like interbands and puffs (designated variously as Early Discontinuous, Interband or Disperse Discontinuous patterns); this early phase is quickly followed by a phase when all disperse and dense chromosome regions are more or less uniformly labelled (the Continuous patterns) and finally the denser bands, intercalary and chromocentric heterochromatin regions continue replication till the very late S-phase (the Discontinuous patterns, see Rudkin, 1972, Hägele, 1973; Roy and Lakhotia, 1979; Mukherjee *et al.*, 1980). In addition to correlating these gross patterns of $^3$H-thymidine labelling of polytene nuclei with their early or late S-phase, such studies also provided a wealth of data concerning the temporal order of replication of specific interband, band, puff sites, etc. (see later). These studies also showed that besides the heterochromatin regions (chromocentric and intercalary), many of the denser bands continued to replicate in late S in a reproducible manner (Plaut *et al.*, 1966; Lakhotia and Mukherjee, 1970; Rudkin, 1972) to generate 'replication bands' analogous to those in the mitotic chromosomes of mammals, etc. Curiously, however, when mitotic chromosomes of *Drosophila* were examined for $^3$H-thymidine labelling or BrdU-Giemsa staining patterns to

analyse the temporal sequence of chromosomal replication, no segmental patterns or 'replication bands' were seen: even with a brief pulse of 10–15 min, the labelled metaphases were uniformly labelled either throughout euchromatin or throughout heterochromatin or throughout both regions (Barigozzi *et al.*, 1966; Kumar, 1979; Steinemann, 1980; Lakhotia 1982; Roy, 1983). Thus, unlike in the polytene nuclei, all the euchromatin regions in mitotic nuclei of *Drosophila* appear to replicate synchronously during the early and mid S period while during the late S, only the pericentromeric heterochromatin blocks replicate. It is possible that some of the later replicating sites in the euchromatin part of *Drosophila* mitotic nuclei may be very small in proportion to the much smaller genome size in *Drosophila* and may thus escape detection in the usual autoradiographic preparations. However, it is significant to note that the mitotic chromosomes of *Drosophila* do not show any G-bands etc. (see Holmquist, 1987 and this volume). Implications of these differences in chromosomal replication of *Drosophila* and mammalian cells are discussed later.

## Patterns of Chromosomal Replication in Differentiated Tissues

The segmental replication patterns of mitotic chromosomes in mammals and of the polytene chromosomes of *Drosophila* encouraged comparative studies on patterns of chromosomal replication of different tissue types of a species. A rather painstaking study in different human tissues was initiated by Prokofieva-Belgovskaya and her group. Employing autoradiography and observing generally the late S, they and certain other groups detected subtle tissue-specific variations in temporal orders of replication among chromosomes (Slesinger and Prokofieva-Belgovskaya, 1968; Slesinger *et al.*, 1974; Prokofieva-Belgovskaya *et al.*, 1976; German and Aronian, 1971; Farber and Davidson, 1977). It was suggested that the basic replication pattern in human cells is that of lymphocytes and the variant patterns superimpose on it as a function of differentiation (Farber and Davidson, 1978). However, autoradiographic studies of Govosto *et al.* (1968) in different hemopoetic cell types in human, and studies on several other mammals (Martin, 1966; Pfluger and Yunis, 1966) could not detect tissue-related differences in the replication order of individual chromosomes. Sheldon and Nichols (1981a,b) employed both autoradiography and BrdU labelling methods to study replication in human as well as avian tissues: they concluded that the minor differences observed in replication within and among different cell types fell within the range of statistical insignificance. Likewise, studies by Epplen *et al.* (1975), employing high resolution BrdU techniques also failed to reveal marked differences among different tissues.

Earlier studies on mitotic chromosome replication generally suffered from the fact that they were mostly carried out during the late S-phase without giving due attention to the duration of the S-phase. A systematic study on the chromosomal replication coupled with measurement of duration of the S-phase in different tissues of two mole rats, *Bandicota bengalensis* and *Nesokia indica*, was carried out in our laboratory (Dubey, 1985). While the length of the S-phase varied among tissues (bone marrow and PHA-stimulated lymphocytes: 13–15h; skin fibroblasts and cells from new born litters: 7.5–9h), the regional order of replication of individual chromosome regions was generally conserved. Nevertheless, due to differences in the length of S-phase more chromosomes replicated at a given time in tissues with a shorter S-phase. It is possible that the differences observed by earlier authors were more a manifestation of differences in the length of S-phase rather than actual differences in the order of replication.

Another approach that has unambiguously demonstrated the permanency of replication patterns has been the study of somatic cell hybrids in which chromosomes of one parental cell line are preferentially lost. A large body of data confirmed that chromosomes in these hybrids retain their parental pattern and that this programme was not altered even if all but one chromosome from one of the parental lines survived in the hybrid (Sonnenschein, 1970; Graves, 1972; Marin and Colletta, 1974; Lin and Davidson, 1975; Farber and Davidson, 1977, 1978).

The above suggests that by and large the replication pattern of each chromosome is determined and regulated autonomously: it is affected neither by the state of differentiation nor by its genomic surroundings. This conclusion, however, needs to be qualified in reference to the late replicating regions of the genome, especially the constitutive heterochromatin and the lyonized X-chromosome in female mammals. Studies on a variety of mammals, including humans, confirm asynchrony of replication between the two X's in female somatic cells. One of them, the genetically active homologue, replicates early in the S-phase, while the other, lyonized and genetically inert, replicates during the later half (Lyon, 1961, 1974). This asynchrony is an ontogenic phenomenon since the two X's replicate synchronously during early S in pre-implantation embryo and the onset of late replication of one X occurs in a differentiation specific manner later in embryogeny. This temporal switch coincides with the formation of sex-chromatin body and genetic quiescence (Lyon, 1974). Recent evidence suggests that an analogous, but not identical, pattern may follow in constitutive heterochromatin in the mouse. Selig *et al.* (1988) have shown that while always replicating in the later quarter of the late S-phase in all body cells of mice, the constitutive heterochromatin replicates during the earlier quarter of the late S in teratocarcinoma of embryonal origin.

In spite of the temporal asynchrony, the regional order of replication in the two X's is generally alike (Schmidt *et al.*, 1982; Camargo and Cervenga, 1982; Reddy *et al.*, 1988). However, whereas the order is almost inviolate in the early-replicating X, it shows considerable heterogeneity in the late-replicating X (Schmidt *et al.*, 1982; Popescu and Dipaolo, 1979) in a tissue-specific manner (Willard, 1977; Cawood, 1981; Reddy *et al.*, 1988). In aneuploid cell lines of female origin, having multiple X's, the heterogeneity among the late-replicating X's is easily marked (Willard and Breg, 1980; Raman, 1984).

Like the facultative X-heterochromatin (lyonized X chromosome), the constitutive heterochromatin on X chromosome also has been found to replicate asynchronously in several rodents. Sen and Sharma (1982) reported that in *Mus dunni*, the constitutive heterochromatin on the active X chromosome in female cells replicates earlier than its homologue on the lyonized X. A comparable situation was noted in the majority of cells of *B. bengalensis* and *N. indica* (Dubey, 1985). Although conclusively demonstrated in only a few species, asynchronous replication of homologous heterochromatin regions appears to be a characteristic feature of X-linked heterochromatin. Temporal heterogeneity in the autosomal heterochromatin has also been recently demonstrated in the mouse (Selig *et al.*, 1988).

The elaborate banding pattern of polytene chromosomes has permitted extensive studies on the temporal order of replication of specific bands, puffs, interbands, etc. in relation to the influence of sex, chromosomal rearrangements and other genotypic factors, developmental conditions and cell types. An important revelation of the early autoradiographic studies on polytene chromosome replication was that the temporal order of replication, particularly during the late S, of specific band regions was

remarkably constant in different wild type strains of *D. melanogaster* and in different laboratories across the world (Rudkin, 1972). However, certain genotypic factors like chromosomal rearrangements, etc., particularly those involving heterochromatin or other late-replicating sites were found to locally alter the replication timings of adjacent regions (Arcos-Terán and Beermann, 1968; Kalisch and Hägele, 1973; Mukherjee *et al.*, 1980; Wargent and Hartmann-Goldstein, 1976; Lakhotia and Mishra, 1982). Developmental conditions or certain mutants that increase the polyteny levels of salivary gland nuclei of late 3rd instar larvae (Hartmann-Goldstein and Goldstein, 1979) also affected the temporal order of replication mainly by prolonging the normally brief early S of a polytene replication cycle (Mishra and Lakhotia, 1982).

Unlike in mammals, the two X chromosomes in polytene cells of *Drosophila* females replicate synchronously with each other as well as with autosomes while the single X in male polytene cells was found to complete its replication earlier than autosomes in the S-phase (Berendes, 1966; Lakhotia and Mukherjee, 1970). This early completion of replication of the X in male polytene cells was correlated with its hyperactivity to achieve dosage compensation (Mukherjee and Beermann, 1965; Lakhotia and Mukherjee, 1969, 1970). It was also shown that parallel with its transcriptional status, the polytene X chromosome in male cells continued its normal faster replication without any effect of or on autosomal segment in autosome-X insertion genotypes (Lakhotia, 1970; Ghosh and Mukherjee, 1986) or in XX/XO mosaic salivary glands (Chatterjee and Mukherjee, 1977). Intriguingly, however, the X chromosome in mitotic cells of male or female *Drosophila* does not show any kind of asynchrony comparable to that noted in polytene nuclei; this aspect will be discussed later (also see Lakhotia and Sinha, 1983).

In spite of the enormously increased resolution offered by polytene chromosome banding pattern, there have been relatively fewer studies on the temporal order of replication of specific chromosome segments in different tissue types. This paucity of studies has been mainly due to the fact that except for the salivary glands of late 3rd instar larvae, the polytene chromosomes found in other cell types in *Drosophila* are of poorer cytological quality and thus unsuitable for such studies. However, in species of *Drosophila* or in certain mutant genotypes of *D. melanogaster*, analysable polytene chromosomes are found in other tissues also and some of these situations have been utilized for comparative studies on temporal orders of replication in different polytene cell types. Lakhotia and Tiwari (1984) and Tiwari and Lakhotia (1984) compared replication of specific chromosome segments in polytene chromosomes of larval salivary glands and gastric caeca in *D. nasuta* and *D. hydei* and found that the independently labelled replication units identifiable at the chromosomal level (see Plaut *et al.*, 1966; Lakhotia and Mukherjee, 1970; Rudkin, 1972) were cytologically comparable in all cases and the units that were late-replicating in one cell type continued to be late-replicating in the other as well. However, within the late S, certain sites displayed characteristically different temporal order of completion of their replication. Recently, Sinha *et al.*, (1987), taking advantage of *otu* mutant alleles in *D. melanogaster* that alter the normally polyneme type of ovarian nurse cells into cells of polytene type (see King and Storto, 1988), compared the replication in larval salivary gland polytene nuclei with that in these nurse cell polytenes. Their results were in general similar to those noted earlier. In a study with polytene chromosomes of the mosquito, *Anopheles stephensi*, Redfern (1981) had also reached a similar conclusion.

The above considerations lead to the following general conclusions: (i) each chromosome has a characteristic temporal and regional order

of replication which is autonomously regulated and (ii) whereas this order is rigidly maintained in the early replicating segments, those replicating in the late S tolerate a certain degree of flexibility both in terms of their temporal and regional orders. It is interesting to note that even among different mammals, chromosomal regions of homologous functions (regions of evolutionary homology) show comparable early S replication patterns (von Kiel *et al.*, 1985).

## Factors Affecting S-Phase—Organization of Active Replicons

It is now well known that the nuclear DNA in eukaryotes replicates in units, the replicons, much smaller than the long DNA molecule that spans even the smallest chromosome. In general, each replicon starts replication bidirectionally from an origin point giving rise to two more or less symmetrically placed replication forks which progress in opposite directions (Hubermann and Riggs, 1968) till they meet forks advancing towards them from the neighbouring replicons or else they encounter a fixed termination site; fusions of replication forks of adjacent replicons leads ultimately to production of two daughter DNA molecules (for reviews see Callan, 1972; Hand, 1978; Taylor, 1978, 1984; Van't Hof and Bjerkens, 1979). There are several possible variables in the organization of eukaryotic replicons: (1) distance between adjacent origin points (replicon size); (2) unidirectional vs bidirectional migration of replication fork; (3) rate of progression of replication fork (rate of replication); (4) fixed termini vs fusion of adjacent forks when they meet; (5) numbers and spatial locations of replicons that are active at a given moment of time in the S-phase (synchrony between replicons of same and different chromosomes in a nucleus). It is these different variables and their subtypes that influence the characteristic patterns of chromosomal replication and duration of S-phase in different species and in different cell types of a species.

Much of the information on these determinants of the S-phase has been obtained using the technique of DNA-fibre autoradiography (Hubermann and Riggs, 1968; Callan, 1972). Some aspects of these will be briefly considered here. Pioneering studies by Callan (1972) and Blumenthal *et al.* (1973) had suggested that the major factor determining the duration of S-phase in a particular cell type of a species was the size of active replicons or the distance between adjacent origin points that are activated to initiate replication since cleavage cells of amphibians and *Drosophila* with very short cell cycles had very closely spaced active origin points while their differentiated cells with longer S-periods utilized far fewer of the potential origin points (also see Taylor and Hozier, 1976; Spradling and Orr-Weaver, 1987). Other studies using human and mammalian cells (Hand and German, 1977; Jagiello *et al.*, 1983; Sung *et al.*, 1986) suggested that the rate of fork migration rather then replicon size determined the length of the S-phase. On the other hand, in the case of plant cells, evidence was presented that a modulation of timing of initiation of replicon families was the critical factor affecting the S-phase duration (Van't Hof, 1975).

Studies in our laboratories, using *Drosophila* as well as mammalian cells (Lakhotia and Sinha, 1983; Lakhotia and Tiwari, 1985; Dubey and Raman, 1983, 1987) have revealed that more than one aspect of the replicon organization is developmentally regulated by different cell types of an organism to attain their characteristic S-phase patterns. In the mole rat, *B. bengalensis*, cell types with S-phase varying from 8h to 14h showed differences both in the rate of fork migration and in replicon size: new-born cells with shorter S-phase had more closely spaced origin points as well as faster migrating forks (Dubey and Raman, 1987). Interestingly, this study also showed that cells with similar

S-phase may also differ in their replicon parameters (size and rate). In an earlier study, Dubey and Raman (1983) had shown that treatment of mole rat cells with Hoechst 33258 affected yet another parameter of replicon activity: the initiation of neighbouring replicon was more asynchronous in treated than in untreated cells.

Studies on replicon organization in polytene (salivary glands) and non-polytene (brain and imaginal disk) cells of *Drosophila* larvae (Lakhotia and Sinha, 1983; Lakhotia and Tiwari, 1985) revealed a novel feature: two very different classes of replicons (designated as Types I and II), differing in their size as well as rate of fork migration, were active in the same cell type during early and late phases of the S, respectively. Moreover, the size and the rate of fork migration in Type I replicons (longer and faster) were found to differ between the polytene and non-polytene cell types analysed (Lakhotia and Sinha, 1983; Lakhotia and Tiwari, 1985).

An important difference in the replicon organization in mammalian and *Drosophila* type systems is the presence of replicon clusters in the former (Edenberg and Hubermann, 1975; Hand, 1978) and their absence in *Drosophila* (Steinemann, 1981a,b; Lakhotia and Sinha, 1983; Lakhotia and Tiwari, 1985). Neighbouring replicons which are activated more or less synchronously constitute the replicon clusters and a mammalian chromosome is constituted of many such clusters. On the other hand, in *Drosophila* chromosomes, entire euchromatin or entire heterochromatin regions appear to constitute single clusters of replicons. The synchrony of replication within clusters seems to be an important aspect of mammalian chromosome organization as reflected in their various banding patterns, etc. (for a more detailed discussion, see Holmquist, this volume). In this context, it is interesting to emphasize differences in the nature of bands of mitotic chromosomes of mammals and polytene chromosomes of *Drosophila*, etc. While their darker staining, species specificity and generally later replication may suggest apparent similarity, a deeper analysis reveals fundamental differences, e.g., each G-band of a metaphase chromosome contains several replicons (replicon cluster), while in *Drosophila* several polytene bands may comprise one replicon. The apparent late replication of many of the polytene bands, giving rise to 'replication bands,' is also a reflection of the complexities associated with replication of the large number of laterally associated sister chromatids of both homologues since as mentioned earlier, mitotic chromosomes of *Drosophila* do not show any later replicating segments or 'replication bands' in their euchromatic regions. To account for the $^{3}$H-thymidine autoradiographic labelling patterns seen at polytene chromosome as well as at their DNA fibre level, Lakhotia and Sinha (1983) proposed that the polytene chromosome organization imposes a temporal and spatial asynchrony in the activation of origin points on homologous replicons of the large number of lateral sister chromatids. This asynchrony leads to continued incorporation of $^{3}$H-thymidine in some of the chromatids while the corresponding replicons in other sister chromatids had already finished and this in turn generates the various segmental or discontinuous labelling patterns, so characteristic of polytene chromosomes but never seen in mitotic chromosomes of *Drosophila*. This asynchrony in activity of homologous replicons on lateral polytene strands also accounts for the overall S-phase being considerably longer than expected on the basis of replicon size and rate of fork migration found in these cells (Lakhotia and Sinha, 1983).

Thus the different possible variables in replicon organization have been utilized in diverse organisms in their various cell types to regulate their S-phases and temporal as well as regional orders of replication of different chromosomes during the S-phase.

## Time of Replication and Transcriptional Activity

Since both the processes of replication and transcription require the same template on which specific polymerases progress to make the respective products, they are expected to influence each other. While the process of replication during a typical S-period makes a global use of nuclear DNA as template, the process of transcription has to be necessarily selective in template usage in a developmentally regulated tissue-specific manner. The specificity of regulated transcription requires a large variety of transient factors binding to the DNA sequences that are to be or not to be transcribed. The process of replication fork migration during the S-period may dislocate these transcription regulatory factors (Brewer, 1988). This obviously necessitates a very intricate organization of transcriptional and replicational activities so that the cell functions proceed unhindered and in orderly fashion. The temporal and regional patterns of chromosome replication are a reflection of this order.

The genes which are required to make their products available in a cell may be expected to replicate early in the S so that the particular DNA sequence is available, without further interruption till the mitosis, for RNA polymerase to act upon. The general temporal exclusion of eu- and hetero-chromatin replication provides a gross compartmentalization of the above type. A finer resolution is seen in the metaphase chromosome band specific replication differences. Stubblefield (1975) in Chinese hamster and others in human cells (Camargo and Cervenka, 1982; Schmidt *et al.*, 1982) have convincingly reinforced the observations obtained from autoradiography of human and muntjac chromosomes that the structural chromosome bands are also distinct in their time of replication in that the early replicating chromosome regions correspond to the R-bands and the late replicating ones to the G-bands. Thus the early and late replicating chromatin appear to be structurally distinct (Korenberg and Rykowski, 1988). Holmquist *et al.* (1982) succeeded in isolating R- and G-band DNA from CHO cells and showed that active and inactive constituents of chromatin were spatially distinct with R-bands harbouring the former, and G-bands the latter. Extending this study further to replication of individual genes, they found a number of house-keeping genes (those active in nearly all body cells) replicating in the early S in different cell types. Among the tissue-specific genes, a strong correlation between their activity and time of replication was established (Goldman *et al.*, 1984). In a series of papers on replication of multigene families, viz., immunoglobulin, globin and histones, Schildkraut and his group established their replication in early S in the tissues of their expression (Furst *et al.*, 1981; Braunstein *et al.*, 1982; Iqbal *et al.*, 1984; Brown *et al.*, 1987). Comparing the replication of functional and pseudogene alleles of globin genes in mice, they found that while the active locus replicated in early S, most of the pseudogenes were late replicating (Calza *et al.*, 1984), thus suggesting that not sequence homology but the expression competence of genes and their location in the genome determines the time of replication in S-phase. Goldman (1988) has chronicled replication timings of all the genes so far studied, and it shows that though the evidence for correlation between early S replication and the expression of a gene is strong, it is not absolute: no gene products has been identified so far for a number of early replicating DNA segments and transcripts from a few late replicating regions have also been obtained.

In the frog, *Xenopus*, there are two sets of 5S-rRNA genes, a cluster of about 800 copies that is expressed in somatic cells (s5S-rRNA) and a much larger cluster expressed in oocytes (o5S-rRNA) but not in somatic cells. In somatic cells, the s5S-rRNA genes replicate in early S

while the o5S ones in late S (Gilbert, 1986; Guinta *et al.*, 1986). In the oocytes also, the o5S cluster is late replicating but is expressed. Hatton *et al.*, (1988) recorded genes in the heavy chain immunoglobulin complex in murine cell lines that were early replicating but were not expressed. However, when expressed, their time of replication was switched to even earlier in the S. Eul *et al.* (1988) fused a lymphoblastoid cell line, in which c-myc expressed and replicated early in S-phase, with lymphocytes and with fibroblasts. The lymphocytic hybrids were tumorigenic while those fused with fibroblasts reverted to a nontumorigenic phenotype. C-myc continued to express and replicate early in the former while in the fibroblastic hybrid, c-myc was unexpressed and late replicating. Thus the correlation between the time of replication and the expression of genes seems to be strong and it may be safe to conclude that in general, replication in early S is a necessary if not essential condition for an expressed gene (Goldman *et al.*, 1984).

The relation between gene activity and time of replication has also been examined in polytene chromosomes. In these chromosomes, the transcriptionally more active regions are distinctly visible as puffs and interbands (Beermann, 1972), and during polytene replication these regions are known to be replicated early (Hägele, 1973; Lakhotia and Roy, 1979; Mukherjee *et al.*, 1980). The early replication of the single X-chromosome in polytene nuclei of male larvae of *Drosophila* has also been correlated with its transcriptional hyperactivity (Berendes, 1966; Lakhotia and Mukherjee, 1970). While these results may suggest a correlation between gene activity and replication time as in mammalian and other cell types, a more critical analysis reveals this correlation between active genes and their early replication in polytene nuclei to be due to certain aspects of polytene chromosome organization rather than this being a general feature of the *Drosophila* genome. As discussed earlier, an interpretation of early and late replication in polytene chromosomes of *Drosophila* is not straightforward in view of the constraints imposed by their unique structural organization. Therefore, it would be proper to look for correlation between early replication and gene expression in non-polytene diploid cell types rather than in endoreplicating ones. The endoreplicated nuclei have multiple copies of most genes on the multitudes of daughter chromatids generated and thus may not perhaps be under the same constraints to regulate time of replication and transcription as the diploid cells. The lateral multiplicity of gene copies allows that, while some gene copies are being replicated, their other homologues may continue to transcribe if the cell requires their transcripts. The model of polytene chromosome replication proposed by Lakhotia and Sinha (1983) provides for such temporal asynchrony in replication of homologous DNA sequences on the lateral strands of polytene chromosomes. If the processes of replication and transcription were mutually exclusive at a given chromosomal location in polytene nuclei, the autoradiographic labelling patterns in $^3$H-uridine labelled nuclei could be expected to be different in nuclei that were replicating and those that were not replicating at the time of pulse labelling. Although this aspect has not been specifically examined, experience shows that progression of replication in a polytene nucleus does not affect its labelling patterns in transcription autoradiograms. From this point of view, the apparent early completion of replication by the X-chromosome in polytene nuclei of male *Drosophila* is not directly and causally related to this chromosome being hyperactive for dosage compensation. As suggested by Lakhotia and Sinha (1983), the earlier completion of the male X-chromosome in polytene cells appears to be due to a greater synchrony in replication of the lateral chromatids of this more loosely organized and hemizygous chromosome: both these conditions pre-

sumably facilitate a greater and quicker availability of the replication machinery to the different lateral subunits. This interpretation finds support in the fact that in mitotic cells of *Drosophila*, the single X in male is not faster replicating. In an interesting study, Hägele and Kalisch (1980) compared replication time of a specific polytene chromosome band before and after puffing (i.e., before and after transcriptional activation) and found that in both situations it continued to be late replicating. Indeed in view of the fact that nearly all replicons in euchromatic regions of the *Drosophila* genome are fired more or less synchronously, the question of correlation between 'early' and 'late' replication of a gene and its expressivity in a tissue becomes a moot point.

## Concluding Remarks

The fidelity and precision of chromosomal DNA replication in eukaryotic nuclei is remarkable considering that the DNA is several thousandfold packed in a typical eukaryotic nucleus and that this highly packed DNA is very orderly and faithfully replicated once and only once every cell cycle. Some of the intriguing questions that arise in this context are: (1) what provides the force and what is the mechanism that permits a rather very rapid unwinding of the double-stranded DNA during replication (and transcription) while it remains as condensed chromatin; (2) what controls the fairly well conserved temporal order of replication of different chromosome regions during an S-phase; (3) what constitutes an origin point of a replicon and how different cell types are able to modulate the use of these origin points with reference to an origin being operative or inoperative in a given cell, and if operative, its time of firing; and (4) how transcription or transcriptional competence affects the replication time of a replicon or replicon cluster. Although clearcut answers to these and related questions are still not available, some understanding of these processes is beginning to emerge with successful development of cell free *in vitro* DNA replication systems and with a better understanding of nuclear matrix and its relation to chromosome structure and organization (Nelson *et al.*, 1986; Gasser and Laemmli, 1987; Blow and Laskey, 1986, 1988; Blow, 1987, 1988; Hubermann, 1987; Bravo *et al.*, 1987; Prelich *et al.*, 1987; Sheehan *et al.*, 1988). It is becoming increasingly clear that the nuclear matrix (or scaffold for metaphase chromosomes) provides a structural framework for attachment of specific chromosomes and chromosome regions. These attachment points not only provide a structural framework for DNA unwinding during replication (and transcription) but also facilitate chromosome and chromatid movements during cell division. In addition, this spatial sub-compartmentalization of chromatin within the nucleus may influence its synthetic activities in other ways also, e.g., by restricting specific chromosomes or chromosome regions to certain functionally distinct spatial domains within the nucleus so that they are or are not available for replication (and/or transcription) at specific times in the cell cycle or in specific cell types. Although not documented conclusively, the nuclear matrix is expected to play a crucial role in defining the early and late replicating chromosomal domains. Thus in the case of late replicating, genetically inert X-chromosomes in female mammals, it is suggested that sometimes during early development, the relegation of one of the two X's to a spatially distinct nuclear location leads to its heterochromatization followed by methylation of its cytosine residues so that the inactive state is cell-inherited (Gartler *et al.*, 1985). It appears that this distinct spatial location is responsible for this X-chromosome becoming late-replicating which in turn leads to its heterochromatization and methylation since in early cleavage embryos of mammals, late replication of one X-chromosome precedes by a few division

cycles the formation of heterochromatic sex-chromatin (Issa *et al*., 1969; Mukherjee, 1976). The constitutive heterochromatin in *Drosophila* is also known to occupy a distinct nuclear location (Lakhotia, 1974; Foe and Alberts, 1985; Hammond and Laird, 1985a,b). An analysis of the 3-dimensional organization of chromosomes in intact polytene nuclei of *Drosophila* also revealed a remarkable order (Hochstrasser *et al*., 1986; Hochstrasser and Sedat, 1987a,b). It is very likely that the specific under-replication of heterochromatin and other sequences in diverse endoreplicating cell types of *Drosophila* is governed by their specific spatial intra-nuclear locations due to interactions with nuclear matrix. An orderly arrangement of chromosomes in a nucleus was suggested more than 100 years ago by Rabl, and a good knowledge of this order and its basis will be essential for understanding chromosomal replication in its totality.

Studies on replication of multigene families (30–250 kb long) in vertebrates showed that the contiguously aligned genes replicate as one replicon (Brown *et al*., 1987) so that replication is regulated in domains larger than individual genes. These larger domains seem to coincide with binding of chromatin at specific points with nuclear matrix through topoisomerase (Gasser and Laemmli, 1987) and with Bal31 cut CHO chromosome fragments of ~90kb length which were considered to represent individual replicons (Clark *et al*., 1987). In this context, it is interesting to note that Dubey and Raman (1987) reported asynchronous replication of contiguous replicons in DNA fibre autoradiograms and which they suggested to be related to active or inactive state of those genes. Thus within a functional domain, a finer regulation of replication is still possible.

In recent years, some progress has also been made in identifying the replicon origins, although the results have often been rather contradictory. Striking similarities were found in origin sequences (*ori* region) of eukaryotic viruses like simian (SV40), polyoma (Py), adeno and Epstein Bar (EPB) viruses. In all of them, the *ori* region comprises a core DNA sequence, the ori sequence, flanked by small DNA motifs; a functional origin requires the entire complex and it is important to realize that the flanking sequences have transcription promoter and enhancer sites which bind with transcriptional and other nuclear factors for initiation and maintenance of replication and transcription (for brief reviews, see DePemphilis and Wassermann, 1987; DePemphilis, 1988). However, the above structure of eukaryotic viral origins is not necessarily ubiquitous for all viral, and even less likely for mammalian replicons. However, the combined organization for regulation of transcriptional and replicational activities does raise interesting possibilities. Significantly, one of the ori sequences recently analysed in HeLa cells, shows some features comparable to those of the viruses, especially in the occurrence of transcription and other nuclear factor binding sites (Triboli *et al*., 1987). Another approach in the study of the origin sequences has been to isolate the autonomously replicating sequences (ARS) from eukaryotic genomes. There are several reports claiming isolation of such sequences. However, none of them have been convincingly shown to be identifiable as a true replicon origin. Thus Thireos *et al*. (1980) identified a region in one of the domains of amplified chorion genes in *Drosophila* as showing ARS activity in yeast; however, this does not coincide with the amplification control element identified for this domain (see Spradling and Orr-Weaver, 1987).

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