Genomic analysis of Drosophila chromosome underreplication reveals a link between replication control and transcriptional territories

Stepan N. Belyakin*, George K. Christophides*, Artyoum A. Alekseyenko*, Evgenia V. Kriventseva†, Elena S. Belyaeva*, Roman A. Nanayev*, Igor V. Makunin*, Heidelberg Fly Array Consortium§, Fotis C. Kafatos†¶, and Igor F. Zhimulev*

*Institute of Cytology and Genetics of Siberian Division, Russian Academy of Sciences, Novosibirsk 630090, Russia; †European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany; and §Zentrum für Molekulare Biologie, Ruprecht-Karls-Universität, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

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In Drosophila polytene chromosomes, most late-replicating regions remain underreplicated. A loss-of-function mutant of the suppressor of underreplication [Su(UR)] gene suppresses underreplication (UR), whereas extra copies of this gene enhance the level and number of regions showing UR. By combining DNA microarray analysis with manipulation of the number of Su(UR) gene copies, we achieved genomic-scale molecular identification of 1,036 genes that are arranged in clusters located in 52 UR chromosomal regions. These regions overlap extensively (96%) but are not completely identical with late-replicating regions of mitotically dividing Kc cells in culture. Reanalysis of published gene expression profiles revealed that genomic regions defined by replication properties include clusters of coordinately expressed genes. Genomic regions that are UR in polytene chromosomes and late replicated in Kc cell chromosomes show a particularly common association with transcriptional territories that are expressed in testis/males but not ovary/females or embryos. An attractive hypothesis for future testing is that factors involved in replication control, such as SU(UR), may interact physically with those involved in epigenetic silencing of transcriptional territories.

DNA replication | polytene chromosomes | suppressor of underreplication | transcriptional silencing

DNA replication in higher eukaryotes is tightly regulated in space and time during the S phase of the cell cycle. The chromosomes are organized into functional domains of DNA replication, the replication foci, within which replication begins simultaneously. Many foci are composed of clusters of replicons, which are considered stable units of the chromosome structure (1, 2). Attempts have been made to correlate specific replication programs with gene expression patterns that may establish the epigenetic chromosomal status (3–6).

The timing of replication in various genomic regions defines them as early-replicated (ER) or late-replicated (LR). Genomic regions that are LR include pericentric and other types of heterochromatin. Early replication strongly correlates with gene activity (3, 5, 7). However, when subjected to position-effect variegation, euchromatic regions that are normally ER become LR (8), and establishment of a LR state during development strongly correlates with gene silencing (8–11).

Polytene chromosomes of Drosophila melanogaster are a unique model for studying replication domains, because of their size and cytological properties and because of the availability of the genome sequence. The size of such domains, their chromosomal distribution, and their genetic and functional organization in specific differentiated cells are matters of substantial interest. Approximately 240 LR regions have been detected in these chromosomes, some of which (≈25% in Oregon-R WT flies) contain weak spots manifested as specific breaks that show incomplete local polytenization [underreplication (UR)]. The LR and UR regions of the chromosomal arms are called intercalary heterochromatin because they share several common features with pericentromeric heterochromatin, including chromatin condensation, frequent ectopic pairing, and location on the inner side of the nuclear envelope (12). However, the information content of these regions has remained unexplored to date.

At least two intercalary heterochromatin regions in polytene chromosomes, 89E and 84AB, contain silenced homeotic Polycomb-dependent genes of the Bithorax and Antennapedia complexes. These chromosome sites are known to contain trimethylated histone H3-K9 (13) and to bind Polycomb-Group silencer proteins (14). Therefore, there are grounds to believe that other intercalary heterochromatin regions may be also genetically silenced (12).

DNA replication in polytene chromosomes depends on the suppressor of UR [Su(UR)] gene (15). UR is suppressed in Su(UR) mutants, Su(UR)−, but augmented in a 4xSu(UR)+ transgenic line carrying two additional copies of the WT Su(UR) allele. Moreover, in the 4xSu(UR)+ line some LR but normally fully replicating regions become UR, demonstrating that extra copies of this gene affect DNA replication, also within those LR regions, which are normally not UR. In addition, detection of SU(UR) protein in LR regions of polytene chromosomes (16) suggests a direct involvement of this protein in both LR and UR. Here, we have exploited these properties of the Su(UR) gene to define UR regions of the larval salivary gland polytene chromosomes at the level of DNA sequences and identified a large set of genes (1,036 or 7.5% of the genome) that are clustered in 52 UR regions. Of these regions, 50 (96%) are also LR in the nonpolyteneic Kc cells, demonstrating a surprisingly consistent timing of replication in two unrelated tissues. Furthermore, a strong link between replication and specific transcription properties has been revealed. We have shown that specific types of transcriptional territories are preferentially located in certain specific types of replication-defined regions of chromosomal arms. Testis-specific territories are associated with UR regions.

Abbreviations: UR, underreplication; Su(UR), suppressor of UR; LR, late replication; LU-R, late but not UR; LfUR, LR-flanking UR; ER, early replication.

1Present address: Harvard Medical School-Partners Healthcare Center for Genetics and Genomics, 77 Louis Pasteur Avenue, Boston, MA 02115.
2Institute of Cytology and Genetics of Siberian Division, Russian Academy of Sciences, Novosibirsk 630090, Russia; †European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany; and §Zentrum für Molekulare Biologie, Ruprecht-Karls-Universität, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany.
3Present address: Harvard Medical School-Partners Healthcare Center for Genetics and Genomics, 77 Louis Pasteur Avenue, Boston, MA 02115.
5Zentrum für Molekulare Biologie Heidelberg, University of Heidelberg, Im Neuenheimer Feld 282, 69120 Heidelberg, Germany; §Division of Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany; Max Planck Institute for Molecular Genetics, Ihnesstrasse 73, 14195 Berlin, Germany; Softberry, Inc., 116 Radio Circle, Suite 400, Mount Kisco, NY 10549; Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany; and Department of Biochemistry, University of California, Riverside, CA 92521.
6To whom correspondence should be addressed. E-mail: kafatos@embl.de.

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and, importantly, these regions lack ovary- and embryo-specific territories. This pattern appears to be associated with gene silencing because it occurs in Kc cells and salivary glands, where testis-specific genes are not expressed, and at chromosomal sites that bind the replication-related Su(UR) protein and one or more known silencing factors.

Materials and Methods

DNA Microarray Hybridization and Analysis. Labeling of genomic DNA was performed according to standard protocols with minor modifications. Three to 5 μg of genomic DNA were digested for 4 h with HaeIII before labeling. Labeled samples were purified through Qiagen (Valencia, CA) PCR purification columns; hybridizations were performed overnight at 42°C in a buffer containing 50% formamide, 6× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), 0.5% SDS, and 5× Denhardt’s reagent (0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% molecular-biology-grade BSA); and washes were carried out at room temperature (twice for 15 min in 0.1× SSC/0.1% SDS and twice for 15 min in 0.1× SSC). Microarray scanning and analysis was performed with the GENEPIX PRO 3.0 scanner and software; data normalization was performed with the GENESPRING software (Silicon Genetics, Redwood City, CA), and data clustering and visualization with the CLUSTER and TREEVIEW programs. We used two different DNA microarray platforms: the first version of the Drosophila Berkeley EST collection (DGC1) and the entire Drosophila gene set (17). The sensitivity of the method to detect UR was first optimized by using the DGC1 platform with total genomic DNA isolated from male and female WT adults (Oregon-R) until we could reliably detect the 2-fold difference for euchromatic genes located on the X chromosome. A second optimization benchmark was provided by the experiments that compared gene dosage in salivary gland of 4xSu(UR)+ males and Su(UR)− females. UR does not occur in the male X chromosome; thus, the comparison revealed a 2-fold difference for X-linked genes in addition to the difference attributed to UR at autosomal loci. The final experiments were performed with microarrays encompassing the entire gene set. Three independent biological samples from females of each strain were assayed. The results were Lowess normalized by using the GENESPRING software (Silicon Genetics), and the reproducibility of the results was checked as described in ref. 18. Features deviating >3 SD (P < 0.01) from the average were not further considered.

Identification of UR Regions. Normalized replication values (ratios of DNA representation in 4xSu(UR)+ vs. Su(UR)− salivary gland chromosomes) of 11,673 genes showing statistically consistent hybridization signals between the three experimental replicates were sorted according to their position in the fly genome (FlyBase Release 3.1 database, available at www.flybase.org). A sliding averaging window of 10 genes, one gene per step, was applied to the normalized data. Resulting values were compared with the average value of all windows in each respective chromosomal arm. Gene windows with values significantly lower (P < 0.05) than the average of that chromosomal arm defined the UR region. Series of overlapping windows were considered part of one UR region, and the outer boundaries of each series were set as the boundaries of the respective UR region. Simulation runs with sliding windows of 5 or 20 genes demonstrated robustness of the applied procedure. LR regions were defined from the original data (7) in a similar manner.

Southern Blot Analysis. Total DNAs from 50 salivary glands and from 25 sets of larval brains and imaginal discs were digested with HindIII endonuclease. DNA was separated in agarose gel and transferred to Hybond-NX membrane (Amersham Pharmacia). DNA fragments were PCR-amplified from genomic DNA, cloned, and labeled with [32P]dATP by random priming. Hybridizations were performed according to the protocol recommended by the manufacturer (Hybond-NX), and blots were exposed for various periods of time at −70°C with Agfa CP-BU x-ray film. Signal intensity was measured by using a Hewlett-Packard Scan Jet 4C/T scanner and the BAND LEADER 3.0 program. Relative DNA abundance was calculated as the ratio of hybridization intensity in salivary glands to imaginal discs after normalization to the rosy gene, which is fully replicated in polytene tissues.

Detection of Transcriptional Territories. The gene expression data of a previously defined developmental data set (19) were initially divided into seven transcriptional programs (see legend of Fig. 4). The relative expression data of each gene (as compared to the standard reference, which was a mixture of all developmental stages) within these programs were averaged, and arithmetic mean values over or below 2-fold were considered indicative of up- or down-regulation, respectively; in-between values were regarded as indicating no regulation. The obtained data were then arranged according to genomic positions, and a sliding nine-gene window (step one gene) across the genome was applied to detect regions enriched in coregulated genes.

Results and Discussion

We used the experimental protocol summarized in Fig. 1 to identify UR regions in the Drosophila polytene chromosomes. Total DNAs prepared from late larval salivary glands of the Su(UR)− mutant strain (where UR is suppressed) and from late larval salivary glands of the 4xSu(UR)+ strain (where UR is enhanced) were labeled with two different fluorophores, respectively, mixed, and hybridized to DNA microarrays containing all predicted genes of the Drosophila genome (17). Comparison of DNAs from those two strains was of utmost importance, as it magnified the UR signal and permitted its unambiguous detection. In the example shown in Fig. 1A, pseudored fluorescence corresponds to UR in 4xSu(UR)+ vs. Su(UR)− DNAs, whereas pseudoyellow fluorescence corresponds to equal levels of polytenization. Three independent experiments were performed, and 11,673 sequences corresponding to unique genes of the fly genome that yielded highly reproducible profiles (P < 0.01) were further processed. By using the genome annotation (FlyBase Release 3.1), we sorted data according to the position of each gene in the genome and generated a whole-genome polytenization profile for the salivary gland chromosomes (Data Set 1, which is published as supporting information on the PNAS web site). We report below the analysis of 52 genomic regions, which encompass genes showing statistically significant (P < 0.05) UR (Table 1, which is published as supporting information on the PNAS web site). As expected, most chromosomal regions, which are always replicated completely, showed similar polytenization levels in Su(UR)− and 4xSu(UR)+ strains.

We validated the microarray-based statistically significant replication profiles by comparing them with corresponding Southern blot-based profiles from three different genomic regions: 19E and 11A on the X chromosome and 89DE on the 3R chromosomal arm (Fig. 1B and data not shown). The profile of region 89DE was reported in ref. 20. The boundaries, length, and main features of the three types of profiles proved quite comparable (given experimental fluctuations and differences in exact coordinates of assessed sequences). The 52 statistically significant UR regions share several common characteristics: (i) they are all located at known cytological sites of LR in the salivary glands cells, (ii) all but one region (35B) were shown previously to colocализ with Su(UR) in WT chromosomes (16), and (iii) their chromosomal positions predominantly coincided with cytologically defined weak spots (which are a morphological criterion of UR). However, some
weak spots were not represented among the 52 UR regions, possibly because of low degree of UR and therefore difficulty of detection, or because of absence of their sequences from the microarrays, if the UR regions are unusually short or predominately intergenic.

We noted a striking coincidence of observed UR patterns in the polytenic salivary glands with recently reported (7) LR patterns in cultured nonpolytenic Kc cells (Data Set 1 and Table 1). Of the 52 UR regions, 50 (96%) also replicate late in Kc cells. Of the 52 UR regions, 50 (96%) also replicate late in Kc cells (Fig. 2A). In other cases, the regions of UR in the salivary gland and those of the LR in the Kc cells overlap but are not coterminal; in such cases, the LR regions flanking an UR region were designated as LR-flanking UR (LFUR) regions (Fig. 2C). Altogether, the data indicate substantial similarity albeit not full coincidence of replication programs among different *D. melanogaster* cell types, polytenic salivary gland cells, and mitotically dividing cultured cells of embryonic origin.

We examined in detail the genetic organization of the most interesting class, the UR regions. These regions ranged in length from 114 to 618 kb and collectively encompassed 1,036 predicted genes or 7.5% of the *D. melanogaster* genes (Fig. 5, which is published as supporting information on the PNAS web site).
Some UR regions contain tandemly duplicated genes. Examples are a cluster of 20 closely related genes encoding a family of transmembrane proteins (\textit{Osiris} cluster or Tpl-locus) (21) at 83DE and a cluster of repeated histone genes located in region 39DE. We tested the overprevalence or underprevalence of Gene Ontology (GO-Slim) terms associated with genes in the UR regions by using the GOTOOLBOX software that is based on a hypergeometric test with Bonferroni correction (22). The analysis detected significant overprevalence of genes with unknown biological function and significant underprevalence of genes involved in biosynthetic processes (both at $P < 0.001$; Table 2, which is published as supporting information on the PNAS web site). However, no clear correlation between UR and gene function could be unambiguously established.

A recent study has shown that 20\% of the \textit{Drosophila} genome is represented by groups of 10–30 adjacent and similarly expressed genes, which are not otherwise functionally related (23). These gene groups were defined as genomic transcriptional territories. Although the 52 UR regions encompass only 7.5\% of the \textit{Drosophila} genome, 30 of them correlate with such transcriptional territories (3-fold enrichment, $P < 0.01$); all are LR in the Kc cells (Table 1). The Kc cell study also reported a link between ER and transcriptional activation (7), suggesting that genes located in LR regions not only become active synchro-
nously but may also be coordinately inactivated in somatic cells. In addition, among the 52 UR regions, 32 (61%) have been previously shown to bind antibodies to Polycomb-Group proteins (Table 1), which repress homeotic gene expression (24). An example is the well known cluster of homeotic genes known as the Bithorax complex, located in 89E. It has been suggested that the presence of silencing complexes may result in condensed chromatin structure, thus delaying and suppressing replication (25).

We inspected our data for possible correlation between replication properties and coordinate gene expression/silencing by using two different expression data sets: the microarray-based developmental data set of expression profiles (19) and an independent data set consisting of genes that are differentially overexpressed (“specific”) in particular tissues or developmental stages, according to subtraction analysis of corresponding cDNA libraries (26). The developmental and cDNA data sets encompassed 3,296 and 5,401 genes, respectively; we have assigned 205 and 451 of these to UR regions, respectively.

By consolidating the developmental expression profiles of genes associated with UR regions, we noted that genes within the same region often show similar expression (Fig. 6, which is published as supporting information on the PNAS website). In particular, genes from UR regions are often up-regulated in males during metamorphosis but not in females (e.g., Fig. 2 and Fig. 7, which is published as supporting information on the PNAS website). Interestingly, these male-specific genes appear to be expressed in the male germ line, because they are no longer expressed in mutants of the tudor gene (19), which plays a key role in male germ line development.

To explore further the possibility that such coordinate gene expression is associated with DNA replication properties, we first mapped the cDNA data set (26) to the four types of regions (UR, LiUR, LU−R, and putative ER regions) in each of the five major chromosomal arms (Fig. 3A). Indeed, this analysis revealed that genes specific for certain tissues or stages (testis, ovary, embryo, larva/pupa, and head) are unequally distributed among these four replication-related types of regions. Statistical tests performed on the original binary (specific/nonspecific) data set using χ² contingency tables showed that the unequal distributions in each of the chromosomal arms except for 3L, and in all five arms combined, were highly significant (P < 0.001). In particular, UR regions (especially in 2L and 2R) are highly enriched in testis-specific genes, compared with each respective chromosomal arm as a whole. In contrast, ovary- and embryo-specific genes are significantly underrepresented in UR regions. Neither the LU−R regions nor the LiUR regions show a similar significant enrichment or underrepresentation.

We mapped in a similar manner the developmental data set (19), which has a much higher temporal resolution although fewer genes. We first averaged the temporal profiles in all four types of regions (same as in Fig. 3A) in each chromosomal arm and in the sum of all five arms combined (black profiles in Fig. 3B). Next, we averaged the profiles in each type of region within a chromosomal arm and in all arms combined and displayed all deviations of these profiles from the respective baseline in red or green for overexpression or underexpression, respectively (Fig. 3B). The UR regions of all chromosomal arms combined showed strong underexpression in embryos and adult females, consistent with the observed deficit of embryo- and ovary-specific genes in the cDNA data set. Conversely, the UR regions showed strong overexpression in males and late pupae, again consistent with the overabundance of testis-specific genes in the cDNA data set. In general, male-specific up-regulation was often accompanied by pupal overexpression (see also Figs. 2A, 6, and 7), consistent with the fact that gonads develop extensively during the pupal stages. These findings were statistically significant at the level of P < 0.001 by Student’s t test. Similar overexpression or underexpression patterns were evident in the URs of individual arms to different degrees: Those in the 3R and X were statistically significant at the same level for embryos, males, and females. In 2L and 2R, similar conclusions could be supported, but at lower levels of confidence (P < 0.01), partly because of the lower number of genes analyzed.

The other three types of regions (LiUR, LU−R, and ER) did not show significant deviations from the baseline in the sum of all arms. However, LiUR regions in 2R and LU−R regions in 2R and 2L showed a similar pattern to UR regions, contrasting with underexpression of LiUR regions in pupae and males in the 2L, 3L, and X chromosomes. These interesting patterns apart of LU−R in 2L were supported at a lower level of statistical confidence (P < 0.05). In summary, the analyses presented in Fig. 3 clearly indicated that developmentally regulated genes tend to cluster in a coordinate manner, in chromosomal regions defined by their DNA replication properties.
The obtained data prompted us to perform more detailed genome-wide analysis of transcriptional territories by using the expression profiles of the cDNA data set (26). We displayed schematically each chromosomal arm and mapped onto it the replication-related related regions and the five categories of specifically expressed genes (Fig. 8, which is published as supporting information on the PNAS web site). In the magnified segment at the end of 2R that is shown in Fig. 4A, two purely testis-specific territories are evident (Fig. 4A, single asterisks), one encompassing the UR 59D and the other located at the distal end of an LU−R, at 60A. Two additional territories (one UR and one LU−R Fig. 4A, double asterisks) also showed enrichment in testis-specific genes and absence of ovary- and embryo-specific genes, although this was less pronounced. In contrast, ER regions encompass genes of all five expression types, some showing internal subclustering. We also analyzed transcriptional territories by using the independent data of the developmental data set (19) and a sliding nine-gene window across the genome. As shown in Fig. 4B, this method confirmed the existence of the four UR and LU−R-associated testis-specific territories that were illustrated in Fig. 4A.

**Conclusion**

This study is a clear example of how genome-wide studies conducted by different authors can be combined by using the genome sequence as the reference framework, leading to unexpected, discovery-driven hypotheses. Here, we have used the unique ability of the SU(UR) protein to modulate UR in *D. melanogaster* polytene chromosomes and thus identified for the first time a large set of genes (1,036 or 7.5% of the genome) that are clustered in 52 UR regions. Most of these (96%) are also LR of testis-specific territories are evident (Fig. 4A, single asterisks), one encompassing the UR 59D and the other located at the distal end of an LU−R, at 60A. Two additional territories (one UR and one LU−R Fig. 4A, double asterisks) also showed enrichment in testis-specific genes and absence of ovary- and embryo-specific genes, although this was less pronounced. In contrast, ER regions encompass genes of all five expression types, some showing internal subclustering. We also analyzed transcriptional territories by using the independent data of the developmental data set (19) and a sliding nine-gene window across the genome. As shown in Fig. 4B, this method confirmed the existence of the four UR and LU−R-associated testis-specific territories that were illustrated in Fig. 4A.

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