Molecular Combing in Studies of the Genome Organization and DNA Replication

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Abstract—Molecular combing (MC) yields preparations where individual DNA molecules are uniformly stretched and are parallel to each other. Fluorescence in situ hybridization on such preparations allows an exact mapping of DNA sequences, and pulsed incorporation of halogenated deoxyuridine analogs and their detection using fluorochrome-conjugated antibodies makes it possible to visualize replication. The MC technique was adapted for studying DNA replication in isolated Drosophila melanogaster organs, and it was checked whether a mutation of the Suppressor of UnderReplication (SuUR) gene directly affected the replication fork rate.

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As was reported in 1994, DNA molecules attach to a regularly moving glass slide under certain conditions, and then they straighten and stretch at the air—fluid interface. The phenomenon provided the basis for a molecular combing technique, which produces preparations of regularly stretched and aligned protein-free single DNA molecules. This makes it possible to physically map particular genome regions on individual DNA molecules at an extremely high resolution. Early experiments on hybridization of cosmid clones to stretched preparations of yeast and human genomic DNAs already showed that sequences size may be mapped up to several kilobases. The method worked well in test experiments with estimating the copy number for chromosome 21 in normal cells and a cell line with trisomy 21 and for an oncogene amplified in the GTL-16 cancer cell line. Thus, MC is suitable for both basic research and diagnosis of genomic alterations associated with cell malignant transformation (for a review, see [1]). While the DNA sequences of euchromatic genome regions were completely established for many organisms, the sequencing of extended repeat clusters is still difficult. MC makes it possible to study the fine organization of such clusters. For instance, it was believed earlier that the human ribosomal gene cluster is a regular repeat of a transcribed region—nontranscribed spacer. It was found, however, that one-third of all rDNA repeats is rearranged to form palindromic structures even in healthy patients regardless of their ages. The proportion of palindromes is increased in patients with a deletion from the WRN gene (Werner syndrome), which codes for a RecQ helicase. Thus, the rRNA genes are organized as a mosaic of canonical and noncanonical (presumably nonfunctional) units, and their proportions may change in the presence of genome-destabilizing factors [2].

A combination of fluorescence in situ hybridization (FISH) with detection of halogenated nucleotides on stretched DNA strands has opened new opportunities for studying DNA replication. Since DNA strands are stretched uniformly, it is possible to estimate the lengths of labeled DNA regions (replication tracks) with a high accuracy. Two-color detection of replication reports the direction of replication fork movement and identifies the positions of replication origins. FISH helps to visualize a DNA region of interest and to study the specifics of its replication. Simultaneous hybridization of two or more probes of different sizes reports the orientation of the region on the preparation. MC has several advantages over other methods that address replication. In particular, MC has a high resolution and makes it possible to compare replication parameters for individual DNA strands and to study the DNA regions that consist of repeats.

Many key studies of the regulation of replication initiation and the epigenetic programming of replication origins employed the MC technique [3–5]. The accumulating results of experiments with stretched DNA strands demonstrate that the replication rate is associated with the number of origins activated in the given cell cycle [6]. In 2008, Nature published an article that substantially clarified the relationship between the replication fork rate and the replication initiation pattern. The replication fork rate was found to corre-
late with the number of origins initiating replication in the same cell cycle in Chinese hamster cells. Delayed replication activates latent origins. Thus, the general temporal pattern of replication is preserved in spite of the changes in replication rate [7]. These findings, which were due to the MC technique to a substantial extent, allowed a fundamentally novel view of the general regulation of DNA replication.

Although significant achievements were made in studies of DNA replication with MC, the method has not been employed in studies with Drosophila as of yet. We were the first to adapt all of the MC steps to estimating the replication parameters in polyploid (salivary glands and fat body) and diploid (imaginal disks and nerve ganglia) tissues of third-instar D. melanogaster larvae (Fig. 1, [8]). After pulsed incorporation of IdU and CldU in isolated organs, nuclei were isolated and embedded in agarose slabs, and genomic DNA was isolated as described in [9]. Glass slides were prepared and MC was carried out according to a published protocol [10]. To obtain preparations of stretched DNA strands, an instrument was designed to ensure even movement of a glass slide in a DNA solution. FISH and immunofluorescence detection of labeled DNA precursors were carried out as in [10]. As an example, Fig. 1 (10) illustrates the mapping of a DNA replication origin close to a histone gene cluster. We measured the replication track lengths on stretched DNA preparations obtained from the salivary glands after 40 min incubation with IdU, and the replication rate in this tissue was thus estimated. The replication rate varied from 0.1 to 0.7 kb/min in wild-type larvae, averaging $0.3 \pm 0.1$ kb/min. According to published autoradiography results, the replication fork rate substantially varies among species and cell types and changes in the course of the S phase in eukaryotes, usually falling in the range from 0.3 to 6.0 kb/min [11]. The average replication rate in cultured D. melanogaster cells was estimated at 0.6 kb/min. In the D. virilis salivary glands, the average replication rate is 0.1 µm/min (corresponding approximately to 0.2 kb/min), threefold lower than in diploid cells of brain complexes [12]. Our analysis of DNA preparations obtained from isolated D. melanogaster organs showed that the replication rate in the salivary glands is, on average, lower than in diploid tissues. Our findings, made with the use of MC, agree well with the published data obtained using autoradiography.

It is known that underreplication of heterochromatic regions in D. melanogaster salivary-gland polytene chromosomes is controlled by the Suppresser of UnderReplication (SuUR) gene. We used MC to check the hypothesis as to whether a mutation of the SuUR gene increases the replication fork rate. The average