DDT Vol. 6, No. 15 August 2001

Targeting the molecular mechanism of DNA replication

Chiara Conti, Sandrine Caburet and Aaron Bensimon

Genome stability is crucial for the complete maintenance of the cellular pathways that govern the cell cycle. As a result of irregularities in DNA replication occurring throughout the S phase, key genes that regulate cell cycle pathways are damaged, giving rise to single-base mutations and chromosomal aberrations. Thus, the efficient replication of the genome, which depends on a precise temporal and spatial pattern of activation of origins of replication, is greatly impaired. The approach discussed below aims at monitoring the replication pattern and the kinetics of replication throughout the entire genome of living cells. It could shed light on the mechanisms by which drugs act on DNA replication and, moreover, it might assist the discovery and design of novel drugs that inhibit cell proliferation under pathophysiological conditions.

Chiara Conti
Sandrine Caburet and
***Aaron Bensimon**
DNA Biophysics Laboratory
Pasteur Institute
25 Rue du Dr Roux
75724 Cedex, Paris
*tel: +33 140 613240
fax: +33 145 688790
e-mail: abensim@Pasteur.fr

▼ Cancer development is a versatile process that arises when the homeostatic balance of a cell is disrupted[1–3]. Tumour progression is then facilitated by loss of fidelity in the processes that replicate, repair and segregate the genome[4]. As a consequence, the genome can accumulate defects that range from single-base mutations to severe chromosomal aberrations[5,6]. The onset of DNA synthesis (S phase) is closely co-ordinated with cell growth, and is tightly regulated by a complex network of interacting factors that signal and control the moment at which the cell is ready for DNA replication. Replication is then initiated at specific sites along the chromosomes once every cell cycle, according to a complex programme[7]. Although important progress has recently been made in understanding the mechanisms that control and co-ordinate cell-cycle progression[8–13], no methods are available for the massive identification, mapping and quantitative analysis of origin firing at the genome level. The knowledge that cancer cells expand by changing their molecular and genetic profiles has led to new and systematic approaches towards the identification and classification of tumour types. This is, in part, a result of significant improvements in molecular cytogenetic methods, which have led to a better understanding of genetic abnormalities. Resolution down to the kb level has been achieved with methods that use different procedures for stretching DNA on a glass surface[14–19]. Some of these techniques, however, are limited by the non-uniform stretching of the molecules and the low number of detectable signals, which lengthen the screening procedure and impede adequate statistical analysis.

There is now a new method, molecular combing, for stretching individual DNA molecules on a solid support. The molecular combing process[20–22] allows genomic changes to be monitored at a resolution range from a few kb to several-hundred kb as the cell cycle progresses. This paves the way for a kinetic analysis of genome instability. This article presents an approach to the global analysis of the replication programme and suggests a procedure for screening lead compounds involved in the regulation of DNA replication, based on single DNA molecule analysis. This route should shed light on the molecular mechanisms of the replication machinery and, hence, facilitate in the testing and quantification of the efficiency of novel compounds on the replication programme of cancer cells and others types of cell.

Molecular combing

Binding and combing single DNA molecules onto a solid surface

Molecular combing is a process whereby DNA molecules, bound by one or both extremities to a solid support, are extended and aligned by the action of a receding air–water interface[20]. The local action of the receding meniscus on

1359-6446/01/$ – see front matter PII: S1359-6446(01)01854-2

the molecules takes place in the immediate vicinity of the interface, thereby enabling constant stretching that is identical for all molecules in solution. The force that the interface exerts on the DNA is strong enough to extend it, but too weak to break the bonds between the molecules and the surface. The stretching forces are also two orders of magnitude greater than the entropic forces that maintain the DNA molecules in their random coil configuration, and is thus sufficient to fully extend the DNA molecules[23]. The extension of a molecule is proportional to its number of base pairs, as expected for homogeneous stretching, and has been measured to be 2 kb μm^{-1} (Ref. 20). This observation also suggests that stretching is not sequence dependent, which is in good accordance with the substantial forces involved in the process.

Binding of DNA to a solid support via its extremities has found many useful applications, most of which require an appropriate modification of the extremities of the molecule. The specific binding of DNA via its unmodified ends can be achieved on a great variety of surfaces by a judicious choice of the pH (Ref. 24). Binding of DNA on hydrophobic and hydrophilic surfaces displays the same behaviour: at low pH, DNA molecules adsorb strongly and non-specifically, whereas at high pH, they adsorb weakly or not at all. In between, there exists a narrow pH range at which DNA strongly and specifically binds to the surface by its extremities. This pH range is typically 0.2 units on hydrophobic surfaces, with the best binding pH varying slightly with surface treatment[24].

Fluorescence hybridization on combed molecules

An important feature of molecular combing is that it ensures the grafting of the molecule at one or both extremities only, avoiding the binding of the molecules throughout its length. Moreover, DNA molecules combed on a glass surface are identically and constantly stretched and retain their structural properties, which permits fluorescence hybridization analysis. Thus, accurate measurements are made on linear fluorescent signals with no need for reference to an internal standard. A technical improvement of the method, referred to as dynamic molecular combing (DMC), has recently been described[21]. This process involves four steps: (1) preparation of suitable surfaces coated with trichlorosilane; (2) preparation of a DNA solution from cells embedded in low-melting agarose blocks; (3) incubation of the surfaces in the DNA solution; and (4) extraction of the surfaces from the solution at a constant speed[25]. The major advantage of DMC is its ability to comb high concentrations of whole genomic DNA in conditions that protect it from extensive shearing, resulting in DNA fragments as long as several-hundred kb (Fig. 1a).

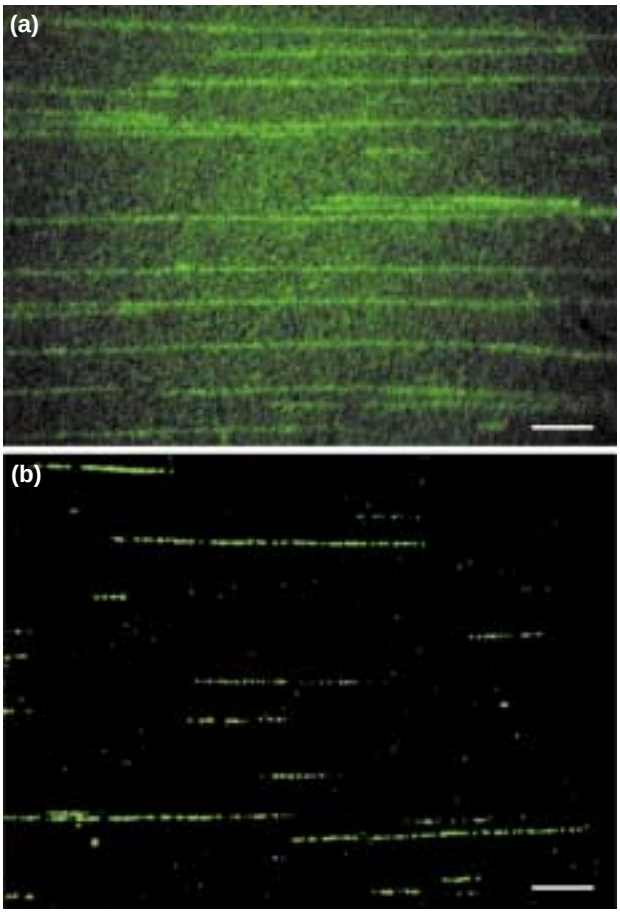

Figure 1. Single molecule analysis of DNA replication. **(a)** Genomic DNA was bromodeoxyuridine (BrdU) pulse-labelled at different time points during the S phase and subsequently combed at low density on a glass surface, yielding well-separated stretched DNA molecules. DNA molecules are visualized and detected with a green fluorescent intercalating agent. **(b)** Combed DNA is detected with a fluorescein isothiocyanate (FITC)-conjugated antibody directed against BrdU. The green segments are DNA molecules into which BrdU was incorporated during the S phase (50 min pulse) – therefore corresponding to replication bubbles. Scale bars, 20 kb.

Fluorescent hybridization of DNA probes on combed DNA allows direct mapping of their respective positions along the fibres[21,26]. Because of the high density of the fibres, scanning and recording of signals is fast. Numerous signals can be measured rapidly, allowing reliable distance measurements by statistical analysis, without reference to any other method or internal control. The reliability of DMC for genomic studies has been shown for the refined physical mapping of the human Ca^{2+}-activated neutral protease 3 gene and, more recently, for the detection of microdeletions in the *BRCA1* gene[27].

Fluorescent hybridization on combed genomic DNA has also been applied to the detection and precise quantification

of amplified sequences ranging from 50 kb to 50 Mb or larger[28]. The aim of this approach is to determine the effective copy number of an amplified region and to compare it with the effective copy number of a non-amplified region. This is achieved by measuring the length of individual hybridization signals from sequence-specific probes. Therefore, the effective copy number of each region is the sum of the lengths of the measured hybridization signal divided by the known length of the respective region. The feasibility of the method was demonstrated by measuring a ratio of 1.5 between the amplified and non-amplified chromosomes in cells carrying a trisomy 21. This approach was later used for the quantification of the proto-oncogene *Met* in two primary human renal cell carcinomas. A first analysis by fluorescent *in situ* hybridization on metaphase spreads had revealed an amplification of the region containing *Met* (Ref. 29). Two and four supplemental copies per haploid genome were found on two successive grafts of these tumour cells established in severe combined immunodeficient (SCID) mice (Conti *et al.*, pers. commun). This indicates an increase in genomic instability during tumour progression.

Visualizing DNA replication from single molecules

DNA replication is a process whereby the activation of origins of replication (ORIs) follows a specific cell-cycle programme[7,30,31]. This programme can vary according to cell type, developmental stage, and internal or external stimuli. A subtle modification of the replication programme can cause severe disruption in the specific spatio–temporal pattern of origin firing. An understanding of the mechanisms that underlie the replication programme at the molecular level is, therefore, essential for the study of cell growth and differentiation, and for the comprehension of genomic aberrations in cancer cells. The capacity to localize and quantify firing of ORIs during the S phase at the genome level aids the study of the kinetics of this process. This requires an analysis of the spatial and temporal organization of replication origins and, specifically, their identification on a genome-wide basis. Classical approaches that are essentially based on two-dimensional gel electrophoresis[32,33] would not allow a massive and quantitative analysis of ORI activities if the whole genome had to be analyzed.

One of many interests in studying DNA replication at the genomic level is to elaborate a complete map of all origins of replication for any given cell type. A comprehensive map of origins and a profile of their activities is essential for the understanding of the replication programme of the respective cell. The experimental approaches used to address these questions require techniques that allow direct and simultaneous visualization of replicating DNA structures from different regions of the genome[34]. Direct visualization allows for measurements to be made on the sizes of these structures and the distances between them. Such measurements provide information about origin location and activity over broad regions of the genome. Because DNA is highly condensed and compacted in the cell nucleus, a key feature of all these techniques is the ability to straighten and label molecules of DNA that contain the replication intermediates. Replicating DNA structures were first visualized by electron microscopy and later by DNA fibre autoradiography. The second technique results in a ‘snapshot’ of individual DNA molecules that undergo replication. More recently, with advances in fluorescent hybridization technologies, several attempts were made to study DNA replication at specific loci on extended chromatin fibres. The goal that motivates the use of these techniques is the determination of the location of origins of replication in a given region of the genome by hybridizing fluorescent probes to the labelled replicated DNA. The advantages of such an approach are obvious: as well as mapping origins along the extended molecule, origin distributions and activities can be conveniently studied under a variety of experimental conditions.

On combed DNA, replication can be directly visualized using immunofluorescence techniques. This involves differential labelling of the replicating DNA with modified nucleotides. Depending on the labelling scheme used, earlier and later replicating sequences in each molecule can be distinguished as alternating green and red fluorescent segments (see Fig. 1, ref. 35). As all molecules on the coverslip are identically stretched, reliable measurements made on the molecules can readily be obtained. This allows for a quantitative assessment of the size distributions of the differentially labelled replication units. These measurements provide an estimate of replicon sizes and, therefore, reflect the replication programme at the level of the genome. On the basis of these measurements, several different analyses can be performed. For example, replication fork density can be determined for any given stage of replication. As the rate of DNA synthesis correlates with fork densities and distributions, the spatial and temporal organization of DNA replication can be directly deduced on a genome-wide basis. Moreover, measurements made on an appropriate set of replication bubbles can reveal the frequency of origin activation during S phase. Assuming a constant replication fork velocity and bi-directional replication, initiation events can also be mapped over time. The reliability of this approach has been demonstrated using the *Xenopus laevis* cell-free system as a model. These experiments demonstrate that the advantage of this method

resides in the ability to obtain a statistically significant number of measurements using a relatively small sample of DNA. This allows for a quantitative analysis of the replication programme in eukaryotes and thus adds to the growing number of techniques now available for elucidating the mechanisms involved in eukaryotic DNA replication[35]. Together, these analyses in turn might reveal correlations between the different parameters that govern DNA replication in a variety of genetic backgrounds and with different drug responses.

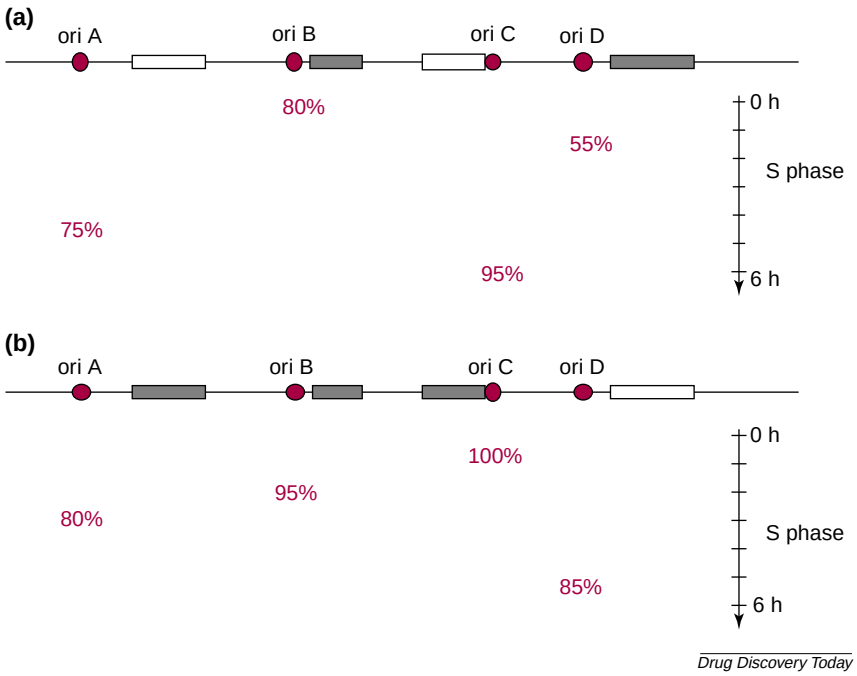

Figure 2. Schematic view of origin activities. Origins of replication (black circles) are first located on a physical map according to their relative position. **(a)** In normal cells, the timing of activation is determined for each origin. As replication and transcription are tightly linked, origins near actively transcribed genes (shaded boxes) are activated earlier than those close to silent genes (open boxes). The number below each origin represents its rate of activation, that is, the percentage of cells for which this origin was actually fired. In the remaining cells, the sequences are passively replicated by a fork from another origin. It is not known whether the early activated origins fire more frequently (i.e. in more cells) than the late ones. **(b)** In cancer cells, the regulation of genes and origins is expected to be disturbed and is, therefore, different from normal regulation. A speculative new replication pattern could be defined, where the same origins are fired at different times and at possibly different rates.

Single molecule analysis of DNA replication: an assay for drug discovery

A major challenge in drug discovery is the elaboration of methods for identifying and testing molecules with therapeutic capabilities in human diseases. Essentially, the process of drug design follows a three-step procedure: (1) identification of the molecular target; (2) development of compounds potentially specific for the target; and (3) elaboration of efficiency and toxicity tests. This approach often uses computer-assisted molecular techniques to design new molecules, because this technique can rapidly analyze hundreds of structures, verify their interaction stability and predict the efficiency of a compound. Nevertheless, in many cases, the three-dimensional structure of the molecular target is unknown at atomic resolution because of difficulties in its isolation, crystallization and crystallographic modelization. Hence, compounds are frequently designed based on structure–function relationships, without precise molecular information on the interactions with the target molecule. Consequently, high-throughput *in vitro* and *in vivo* screening for the therapeutic activity of potential drugs remains the most important step in drug discovery, particularly for cancer drugs.

As discussed later, molecular combing can serve as a tool for the rapid high-throughput determination of the efficacy of lead compounds assigned to attenuate pathophysiological cell proliferation. In conjunction with other methods, it is a particularly appealing approach to use for investigating those factors involved in specifying and determining origin location and activities. In metazoan cells, origins have been difficult to identify because of the lack of a consensus origin sequence. A widescale approach to localizing pulse-labelled replication units within specific regions of the genome, followed by mapping of the corresponding origins using higher resolution techniques, can be envisaged. This will permit the direct cloning of specific replication origins, and the identification of autonomously replicating sequences and other *cis*-acting determinants of origin function. In this manner, for example, elucidating the complete replication profiles that correspond to a tissue-specific replication programmes might now be feasible.

The same principle can be applied to test whether a novel lead compound can affect the replication machinery at the molecular level. For this purpose, the specific pattern of activation of origins of replication must be defined in normal cells. This could then be used as a standard and compared with the displayed pattern in cells that were subjected to drug treatment (Figs 2 and 3a,b). Molecular parameters that

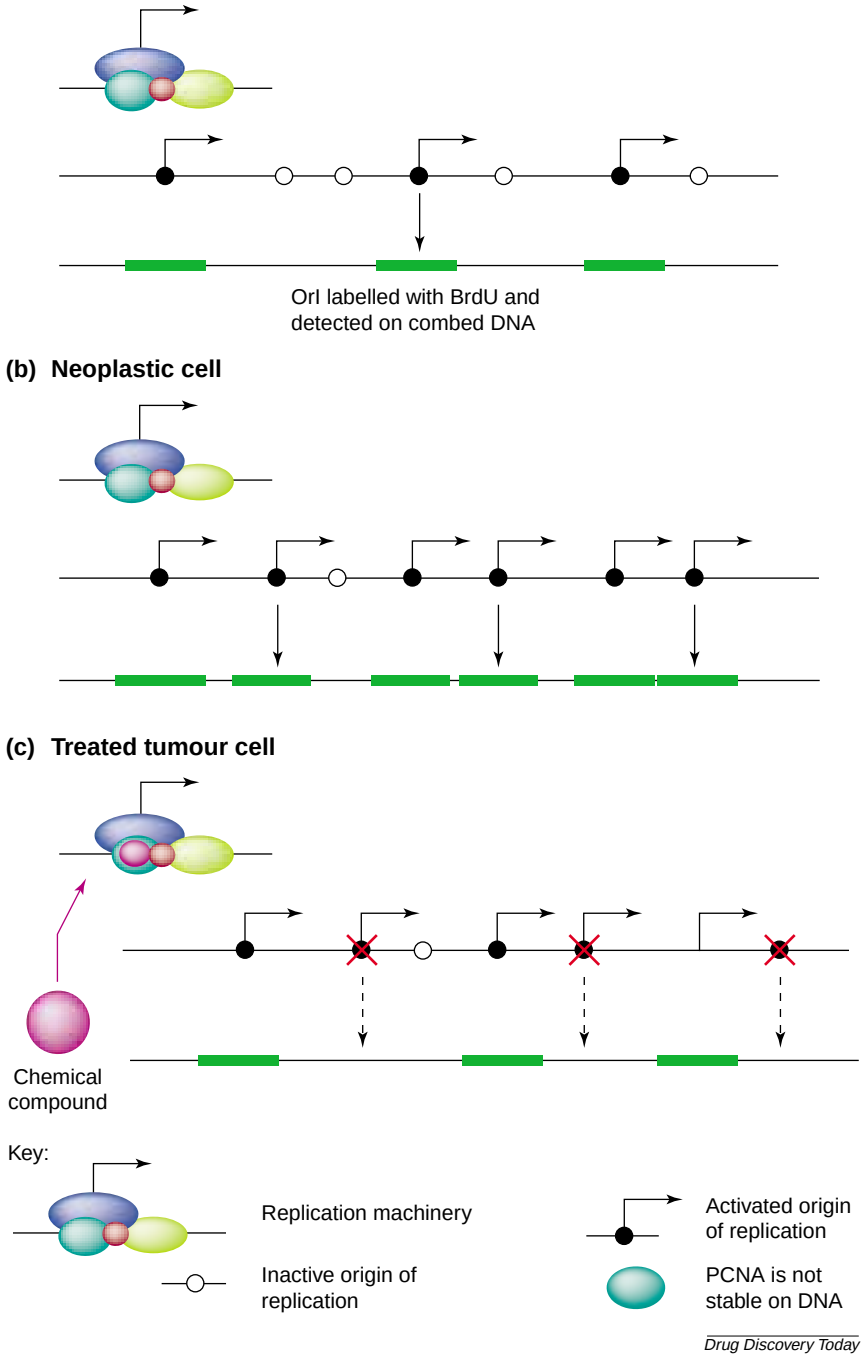

Figure 3. Spatial order of activation. The activation of origins of replication is tightly regulated. Only part of the pool of origins of a given chromosome is activated. **(a,b)** The assumption is that normal and neoplastic cells display different patterns of activation. According to the approach explained in Fig. 1, the firing of an origin results in a replication bubble that is labelled and detected as a fluorescent green signal. Once activated origins have been found in a normal cell, they can be compared with those in a neoplastic one. **(c)** It is then possible to assess the efficiency of a chemical compound. When directed against an element of the replication machinery, an adequate drug should re-establish the normal pattern in a transformed cell. In the example shown, the efficiency is measured as the number of supplemental activated origins that are inhibited by the compound.

govern DNA replication, such as fork velocity, fork density and frequency of initiation, could be compared and would provide information about molecular changes that occurred as a function of drug treatment. The dose–response to different drugs and lead compounds could be followed precisely by monitoring the changes in these parameters. As the effect of drugs on the molecular machinery of DNA replication could be quantitatively measured, one could attempt to search for drugs that would re-establish a normal replication programme in cancer cells (Fig. 3c). Moreover, several successive cell-cycle checkpoints are known to maintain the state of a normal cell[36]. Processes that lead to cancer are based on the ability of the cell to bypass those cell-cycle checkpoints; however, their precise function is sometimes unclear. Measurements made on the molecular parameters of DNA replication, for example, fork velocity, fork density, and frequency and timing of initiation, would thus shed light on the mechanisms involved in carcinogenesis and would constitute the basis for more specific drug discovery-orientated research.

Chemotherapy, the conventional treatment for cancer, mainly uses drugs that block the S phase, the G2 or M phase and regulatory pathways controlling cell-cycle machinery. The major drawbacks of chemotherapeutic compounds are: (1) their lack of specificity; and (2) their severe toxicity in normal tissues. Single-molecule analysis of DNA replication could, therefore, be used for the analysis of drugs that are principally directed against DNA and DNA-processing enzymes. Molecules that affect DNA and induce cell-cycle arrest can be divided into two classes: (1) compounds that induce cell arrest at the G1 or S phase by affecting the nucleotide pool of the cell or by damaging enzymatic reactions involved in DNA synthesis (e.g. antifolates, 5-fluoropyrimidines, cytidine analogues,

purine antimetabolites, hydroxyurea and aphicolidan)[37]; and (2) compounds that directly act on DNA molecules, like DNA alkylating compounds[37] and platinum derivatives, which form a strong complex with guanine and adenine. Other compounds, such as anthracyclines, epipodophyllotoxins and ellipticine derivatives, inhibit DNA topoisomerase II, a pivotal enzyme in releasing torsional strain in DNA, and arrest the cell cycle in G2 phase. The effects of these molecules on the molecular parameters of DNA replication could thus be precisely measured. This would help to define new approaches for the selection of drugs and would provide adequate information regarding the toxicity and specificity of these compounds.

An accurate coordination of gene expression and DNA replication is crucial for cell survival. Microarray analyses for gene expression provides large amounts of data regarding the changes in the expression profiles of thousands of genes in different cellular environments. The rationale behind such analyses is to select key genes involved in cellular processes, such as cancer, to better understand their mode of action. Indeed, the replication programme differs from one tissue type to another, from one stage of development to another and from normal cells to cancer cells, depending on the respective transcription profile of the cell. Hence, molecular combing could have an impact by establishing an integrated replication/transcription map of different regions of the genome at different stages of development or during carcinogenesis.

Conclusions

We have reviewed the molecular combing method for stretching single DNA molecules on a solid support. Molecular combing was first used for the detection of genomic rearrangements, including amplifications and deletions. The method has been successfully applied to the clinical diagnosis of genomic instability in human cells. For example, rearrangements in the *BRCA1* gene have been detected by a colour barcode strategy in the genomes of several individuals[27]. Genomic instability at the *Met* locus could also be monitored over time in renal carcinoma, during tumour progression (C. Conti *et al.*, unpublished).

A further application of molecular combing has been achieved by studies of the kinetics of DNA replication. Results obtained, for both the *in vitro* cell-free system of *X. laevis* egg extracts[35] and yeast cells[38], show the feasibility of the approach and is currently being tested on human cells. The replication profile, defined as the spatial and temporal pattern of origins activation must be established in a control system to constitute a standard for further studies on different genetic backgrounds. Both a local approach, which focuses on a specific region of the genome, and a global approach, which provides a broader view of the kinetics of DNA replication, are of interest for the understanding of drug action on the mechanisms that govern the cell cycle. The information regarding the position of origins of replication is required for the local approach, but other parameters, such as fork velocity, fork density and initiation rate, are taken into account and provide global information on the whole genome.

We suggest that this approach would be used at the later stages of drug research for testing lead compounds that affect the replication process. Some compounds that are directed against DNA and DNA-processing enzymes and have an impact on DNA replication are readily available. However, drawbacks that can be investigated by molecular combing still remain, for instance, the dose that has to be administered *in vivo* of a given drug without conferring toxicity. New synthetic compounds can also be tested and, more generally, any deviation from the replication standard can be studied at the molecular level by the approach presented here. Differences between tissues or embryonic developmental stages can also be investigated, by using microarrays for a more comprehensive survey.

An approach via molecular combing presents two technical drawbacks. First; limitation of resolution because of the use of epi-fluorescence optical microscopy [point mutations and small rearrangements (under 1–3 kb) cannot be detected]. The second limitation lies in the time-consuming analysis of each slide. Automation (under development) is necessary, therefore, to enable use of the method for high-throughput analyses. Once automation is set up and validated, the approach could be used as a diagnostic tool in drug discovery and clinical trials.

Acknowledgement

The authors' projects are partially supported by an NIH grant.

References

1 Gray, J.W. and Collins, C. (2000) Genome changes and gene expression in human solid tumors. *Carcinogenesis* 21, 443–452
2 Orr-Weaver, T.L. and Weinberg, R.A. (1998) A checkpoint on the road to cancer. *Nature* 392, 223–224
3 Loeb, K.R. and Loeb, L.A. (2000) Significance of multiple mutations in cancer. *Carcinogenesis* 21, 379–385
4 Schar, P. (2001) Spontaneous DNA damage, genome instability, and cancer – when DNA replication escapes control. *Cell* 104, 329–332
5 Lengauer, C. *et al.* (1998) Genetic instabilities in human cancers. *Nature* 396, 643–649
6 Cahill, D.P. *et al.* (1999) Genetic instability and Darwinian selection in tumours. *Trends Cell Biol.* 9, M57–60
7 Zannis-Hadjopoulos, M. and Price, G.B. (1998) Regulatory parameters of DNA replication. *Crit. Rev. Eukaryotic Gene Expr.* 8, 81–106
8 Hartwell, L.H. and Kastan, M.B. (1994) Cell cycle control and cancer. *Science* 266, 1821–1828

9 Navas, T.A. *et al*. (1995) DNA polymerase epsilon links the DNA replication machinery to the S phase checkpoint. *Cell* 80, 29–39
10 Morgan, D.O. (1995) Principles of CDK regulation. *Nature* 374, 131–134
11 Bodnar, A.G. *et al*. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* 279, 349–352
12 Paulovich, A.G. *et al*. (1997) When checkpoints fail. *Cell* 88, 315–321
13 Paulovich, A.G. *et al*. (1997) RAD9, RAD17, and RAD24 are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. *Genetics* 145, 45–62
14 Heng, H.H. *et al*. (1992) High-resolution mapping of mammalian genes by *in situ* hybridization to free chromatin. *Proc. Natl. Acad. Sci. U. S. A.* 89, 9509–9513
15 Wiegant, J. *et al*. (1992) High-resolution *in situ* hybridization using DNA halo preparations. *Hum. Mol. Genet.* 1, 587–591
16 Parra, I. and Windle, B. (1993) High resolution visual mapping of stretched DNA by fluorescent hybridization. *Nat. Genet.* 5, 17–21
17 Fidlerova, H. *et al*. (1994) Two simple procedures for releasing chromatin from routinely fixed cells for fluorescence *in situ* hybridization. *Cytogenet. Cell Genet.* 65, 203–205
18 Senger, G. *et al*. (1994) Released chromatin: linearized DNA for high resolution fluorescence *in situ* hybridization. *Hum. Mol. Genet.* 3, 1275–1280
19 Houseal, T.W. *et al*. (1994) High resolution mapping of overlapping cosmids by fluorescence *in situ* hybridization. *Cytometry* 15, 193–198
20 Bensimon, A. *et al*. (1994) Alignment and sensitive detection of DNA by a moving interface. *Science* 265, 2096–2098
21 Michalet, X. *et al*. (1997) Dynamic molecular combing: stretching the whole human genome for high-resolution studies. *Science* 277, 1518–1523
22 Herrick, J. and Bensimon, A. (1999) Imaging of single DNA molecule: applications to high-resolution genomic studies. *Chromosome Res.* 7, 409–423
23 Bensimon, D. *et al*. (1995) Stretching DNA with a receding meniscus: Experiments and models. *Phys. Rev. Lett.* 74, 4754–4757
24 Allemand, J.F. *et al*. (1997) pH-dependent specific binding and combing of DNA. *Biophys. J.* 73, 2064–2070
25 Conti, C. *et al*. Molecular combing. *Curr. Prot. Cytometry* (Vol. 1 Unit 8.10, Suppl. 16)
26 Weier, H.U. *et al*. (1995) Quantitative DNA fiber mapping. *Hum. Mol. Genet.* 4, 1903–1910
27 Gad, S. *et al*. (2001) Color bar-coding the BRCA1 gene on combed DNA: a useful strategy for detecting large gene rearrangements. *Genes Chromosomes Cancer* 31, 75–84
28 Herrick, J. *et al*. (2000) Quantifying single gene copy number by measuring fluorescent probe lengths on combed genomic DNA. *Proc. Natl. Acad. Sci. U. S. A.* 97, 222–227
29 Glukhova, L. *et al*. (1998) Overrepresentation of 7q31 and 17q in renal cell carcinomas. *Genes Chromosomes Cancer* 22, 171–178
30 dePamphilis, L. (1996) Origins of DNA replication. In *Concepts in Eukaryotic DNA Replication*, (Chapter 2) pp. 45–87, Cold Spring Harbor Laboratory Press
31 Simon, I. and Cedar, H. (1996) Temporal order of DNA replication. In *Concepts in Eukaryotic DNA Replication*, (Chapter 12) pp. 387–408, Cold Spring Harbor Laboratory Press
32 Brewer, B.J. and Fangman, W.L. (1987) The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* 51, 463–471
33 Huberman, J.A. *et al*. (1987) The *in vivo* replication origin of the yeast 2 microns plasmid. *Cell* 51, 473–481
34 Blumenthal, A.B. *et al*. (1974) The units of DNA replication in *Drosophila melanogaster* chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 38, 205–223
35 Herrick, J. *et al*. (2000) Replication fork density increases during DNA synthesis in *Xenopus laevis* egg extracts. *J. Mol. Biol.* 300, 1133–1142
36 Elledge, S.J. (1996) Cell cycle checkpoints: preventing an identity crisis. *Science* 274, 1664–1672
37 Damiens, E. (2000) Molecular events that regulate cell proliferation: an approach for the development of new anticancer drugs. *Prog. Cell Cycle Res.* 4, 219–233
38 Lengronne, A. *et al*. (2001) Monitoring S phase progression globally and locally using BrdU incorporation in TK(+) yeast strains. *Nucleic Acids Res.* 29, 1433–1442