

Asynchronous DNA replication detected by fluorescence *in situ* hybridisation as a possible indicator of genetic damage in human lymphocytes

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Abstract. The present study aimed to correlate the DNA replication timing of different genes with genetic damage and frequency of cancer. Using a fluorescence *in situ* hybridisation (FISH) approach, the replication timing of three loci, two human genes possessing transcriptional capability and involved in both the cellular response to genetic damage and cancer development (*TP53* and *RBI*) and the non-coding locus *D22S163*, was evaluated. The data obtained show that normal human lymphocytes exposed *in vitro* to known DNA-damaging agents, e.g. H₂O₂, ionizing radiation and mitomycin C, exhibit an asynchronous replication of the genes *TP53* and *RBI*. *In vivo* studies were performed in three different populations from Kazakhstan. In two of these populations that are living in polluted areas and have higher cancer mortalities than people living in a control area, a DNA replication behaviour similar to that observed in human lymphocytes exposed *in vitro* to known genotoxic agents was detected. The results obtained further indicate that DNA damage hampers replication and FISH represents a fast and accurate method of assessing asynchronous replication by providing an important tool to evaluate DNA damage at a populational level.

Introduction

Kazakhstan suffers from substantial pollution problems with consequential environmental impact. Numerous environmental agents are a threat to human genetic health. In particular,

radiation and some chemicals may lead to genetic changes in human cells that are considered an etiologic factor of cancer. Although the mortality rate for cancer has fallen in recent years in that country, it is one of the highest compared to other Central Asian countries and Europe (1). Two of the major objectives in population cancer risk evaluation are the increase of the accuracy of exposure assessment to potential environment carcinogens and the prediction of cancer outcome in exposed populations (2).

With respect to cytogenetic methods, the detection of unstable chromosome aberrations is the method of choice for most suspected cases of accidental overexposure when the analysis can be performed within months after exposure, but is unsuitable when a period of years has elapsed after the exposure because of the limited lifespan of unstable aberrations. The stable chromosome aberrations are observed after conventional banding alone or in combination with FISH methods, but these techniques are laborious and time-consuming (3).

The development of simple, rapid and reliable assays is thus desirable in order to estimate the genetic damage and predict its consequences in human populations.

Replication timing studies have been used to monitor early and late replication, showing an altered replication phenotype in human cells with chromosomal aberrations (4,5). These authors show that certain chromosomes carrying translocations display significant delayed replication timing, while a derivative human chromosome containing a homogeneously staining region (HSR) is primarily early replicating. Early replication timing has also been reported in association with the expression of both the *RET* gene (6) and the translocated *BCL-2* gene (7).

Recent evidence has suggested a number of interrelationships between DNA replication and cellular DNA damage responses (reviewed in ref. 8). In fact, Merrick *et al* (9) observed altered replication dynamics after DNA damage in human cells, showing that exposing S-phase cells to ionizing radiation induces a transient block to origin firing but does not affect the fork rate or fork stalling.

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Fluorescence *in situ* hybridisation (FISH) analysis could be used on interphase nuclei to determine the replication timing of DNA (10-12). This assay has many parallels with the assay developed by Woodfine *et al* (13) using genomic clone DNA microarrays to assess the replication timing of sequences during the S phase of the cell cycle. However, the FISH analysis presents an advantage on the detection of the DNA replication asynchrony which could not be detected by the approach described by Woodfine *et al* (13) since the ratio reported by these authors is a combination of the replication timing of the two alleles. Using FISH methodology it was shown that lymphocytes derived from patients with haematological malignancies (14), renal carcinoma (15) and neurofibromatous type 1 (16) display a loss of synchrony in allelic replication timing.

The present study was primarily aimed at assessing replication asynchrony in populations with a high mortality rate of cancer due to their living in polluted areas.

Two human genes possessing transcriptional capability and involved in both the cellular response to genetic damage and cancer development (*TP53* and *RBI*) and the non-coding locus *D22S163* were studied by a FISH-based approach to determine the timing of replication. Replicated loci are visualized as double-dot hybridisation signals (biallelic replicated state), whereas the unreplicated loci are visible as single dots. Genes undergoing non-synchronous DNA replication appear as one singlet and one doublet in the interphase nuclei (monoallelic timing of replication). Thus, the allelic counterparts replicating asynchronously give rise to a high frequency of cells with one singlet and one doublet (SD cells). By scoring at least 200 cells one can assess whether a gene is early, late or asynchronously replicating.

In order to optimise the FISH method for further application to the populations described below and calibrate the frequency of cells with asynchronous replication of the *TP53* and *RBI* genes, cells from healthy non-smoker volunteers, who were not exposed to radiation or drugs, were treated *in vitro* with ionising radiation, mitomycin C or hydrogen peroxide. A FISH analysis and the parallel scoring of chromosome aberrations in metaphase spreads were carried out in the same aliquots of cells.

The replication timing of the three loci was investigated in populations living in two polluted areas of Kazakhstan, Ust-Kamenogorsk and Aktau (Mangistau area, Kora lake). Volunteers from the Almaty area (Chylik) served as controls. Populations living in the Ust-Kamenogorsk area have a high mortality rate from cancer of various organs (Table I) whereas populations from Aktau have a high mortality rate of oesophagus and colon cancer and leukaemia as compared in both cases with the control region of Almaty.

Materials and methods

Subjects. For *in vitro* exposure to genotoxic agents, blood was withdrawn by venipuncture from 7 healthy Portuguese volunteers who were non-smokers and without recent exposure to drugs or radiation.

In vivo studies were performed in three populations from the Kazakhstan areas of: i) Ust-Kamenogorsk, ii) Aktau (Mangistau area, Kora lake) and iii) Almaty (Chylik). The

Table I. Tumour sites for which there is an increased mortality rate in Ust-Kamenogorsk as compared to the Almaty area (per 100,000 inhabitants, in the year 2000).

Organs	Ust-Kamenogorsk area	Control group (Almaty area)
Mouth and throat	4.1	2.4
Stomach	34.0	14.3
Colon	13.0	2.8
Rectum	11.4	3.6
Lung	44.2	16.4
Bones	4.7	2.3
Skin	2.2	0.8
Breast	14.3	7.2
Ovary	5.4	2.7
Prostate	3.8	1.7
Urinary bladder	4.7	1.7
Lymphoma	5.0	1.7
Leukemia	5.0	2.2

first two populations are living in polluted areas and have a high mortality from cancer and the third is a control population. The data on cancer mortality was obtained from the Laboratory of Scientific Management in the Fight Against Cancer and Application of the Kazakh Scientific Research Institute of Oncology and Radiology, Ministry of Health of the Republic of Kazakhstan. The tumour sites for which there is an increased mortality in the Ust-Kamenogorsk area compared with the control area is indicated in Table I. In the Aktau (Mangistau area, Kora lake) region the total mortality rate from cancer in 2000 was similar to the Almaty control area (96.9 vs. 100.6 per 100 000). However, the oesophagus and colon cancer and leukaemia had higher mortality rates in Aktau, respectively, 15.6 vs. 7.7, 4.1 vs. 2.8 and 4.4 vs. 2.2.

Cell suspensions in fixative were received from 24 inhabitants from the Ust-Kamenogorsk area, 25 inhabitants from Aktau (Mangistau area, Kora lake) and 20 inhabitants from the Almaty area (Chylik).

***In vitro* exposure to genotoxic agents.** For irradiation, the blood was exposed to X-rays (1 and 2 Gy) using a Varian linear accelerator model Clinac 2100 (C/D). Non-irradiated blood was treated with mitomycin C (0.3 and 1.5 μ M) or H₂O₂ (15 and 20 mM) at 24 h of culture. These doses were chosen since they produce a significant induction of genetic damage (17).

Cell cultures. Aliquots of 0.5 ml of heparinized whole blood were cultured in 4.5 ml Ham's F10 medium supplemented with 24% foetal calf serum, penicillin (100 IU/ml), streptomycin (100 μ g/ml), 1% L-glutamine and 1% heparin (50 IU/ml). Lymphocytes were stimulated using 25 μ l of phytohemagglutinin (PHA) and incubated at 37°C for 48 h. Two hours before harvesting the cells, 0.1 ml of a 4 mg/100 ml solution of colchicine was added. Cells were harvested by

centrifugation, submitted to hypotonic treatment and fixed with cold methanol : acetic acid (3:1). Cells were spread on slides and stained with Giemsa or stored at -20°C for further use in FISH analysis. Preparations for FISH were made by applying one or two drops of cell suspension on silanized slides.

Probes. For hybridisation, labelled commercial probes obtained from Vysis, Inc. were used for the three loci studied: the *TP53* probe (32-190004), *RB1* probe (32-190001) and *MS607* probe (33-260022), a minisatellite probe from locus *D22S163*. The probe mixtures were prepared according to the manufacturer's instructions.

In situ hybridisation. Preparations were dehydrated in 100% EtOH for 5 min, denatured for 1.5 min in 70% formamide/2X SSC (pH 7.5) at 73°C and dehydrated again in a graded ethanol series. Probe hybridisation was performed for 20 h at 37°C in a moist chamber. Slides were then washed following the manufacturer's protocol and counterstained with DAPI (Vysis).

Cytogenetic evaluation. Slides were analysed on a Nikon Optiphot microscope coupled to a CytoVision system from Applied Imaging. Cy3 and DAPI filters were used. The DNA replication timing evaluation was done whenever possible in 200 nuclei for each individual and each probe. The same was done in the *in vitro* study for each dose of each genotoxic agent. Scoring was only done when the following criteria were met: i) nuclei had intact morphology, ii) nuclei did not overlap and iii) both hybridisation signals were visible. As illustrated in Fig. 1, non-replicated sequences were detected as two single hybridisation signals (SS) and sequences that have a completed replication were detected as two double hybridisation signals (DD). Sequences undergoing non-synchronous DNA replication appeared as one singlet and one doublet (SD).

To test for hybridisation efficiency, metaphase spreads were examined for the number of hybridisation signals at each chromosome copy. The level of SDs attributed to incomplete hybridisation was 10% at maximum, consistent with previous reports (18).

The scoring of chromosomal aberrations was assessed according to the criteria described by Rueff *et al* (17).

Statistical analysis. In order to analyse the data *in vivo* the one-way ANOVA was used to check if differences between the cities in the study existed. The Scheffé method was used in the cases where differences were found to exist, to select the pairs of cities, which differed significantly at the 5% level. The statistical analysis of the homogeneity of age (<30, 30-39, 40-49 and ≥ 50 years), gender and smoking habits (smokers and non-smokers) and distributions between exposed and control populations was carried out using the χ^2 test.

For the data *in vitro*, two-way ANOVA was used to check the existence of differences between the treatments. The Scheffé method was again used. The Dunnett method was used in order to detect if the treatments were significantly higher or lower than the controls. The correlations of different variables were determined by the Pearson correlation test. The

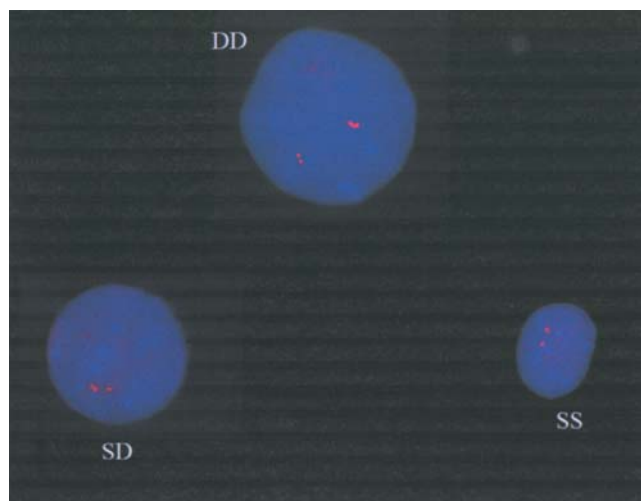


Figure 1. Hybridization signals of replication, using three different DNA probes: *TP53*, *RB1* and *MS607*. (DD) A nucleus exhibiting two double hybridization signals (DD), obtained with a *TP53* probe. (SD) A nucleus exhibiting one single and one double hybridization signal (SD), obtained with an *RB1* probe. (SS) A nucleus exhibiting two single hybridization signals (SS), obtained with an *MS607* probe.

differences were considered significant at the 5% level for all the analyses performed.

Results

DNA replication timing in normal human lymphocytes exposed to genotoxic agents (in vitro study). The frequency of the SS cells for *TP53* and *RB1* in peripheral blood lymphocytes prior to exposure to the genotoxic agents was 81 ± 6 and $84 \pm 8\%$ (mean and standard deviation). The frequencies of the SD and DD cells were low, 13 ± 4 and $7 \pm 2\%$ for *TP53* and 9 ± 5 and $7 \pm 5\%$ for *RB1*, indicating the synchronous replication of the *TP53* and *RB1* genes. However, after cell culture exposure to X-rays (2 Gy) the corresponding values of the SS cells decreased to 65 ± 4 and $61 \pm 5\%$ for *TP53* and *RB1*, respectively (Figs. 2 and 4). For both the *TP53* and *RB1* genes, the decrease of the SS cells and the increase of the SD cells caused by the X-rays (2 Gy) were statistically significant ($P < 0.05$), indicating that one of the alleles of each locus replicated earlier than the other allele (Figs. 2 and 4). After the exposure to a lower dose of ionising radiation (1 Gy) a statistically significant increase of SD and DD cells was observed when the *RB1* probe was used ($P < 0.05$, Fig. 4). Comparing these effects with the induction of chromosomal aberrations, we could detect a significant Pearson correlation between dicentric formation and the frequency of SD cells when the *RB1* probe was used ($r = 0.999$, $p = 0.026$). A similar correlation was observed when the *TP53* probe was used but this did not reach the significant level.

Mitomycin C (0.3 and $1.5 \mu\text{M}$) also led to an asynchronous replication evidenced by the significant decrease of the SS cells and the significant increase of the SD cells after treatments ($P < 0.05$). Using the *TP53* probe, the frequency of the SS cells was 59 ± 11 and $66 \pm 10\%$ and the frequency of the SD cells was 25 ± 8 and $26 \pm 12\%$ after exposure to 0.3 and

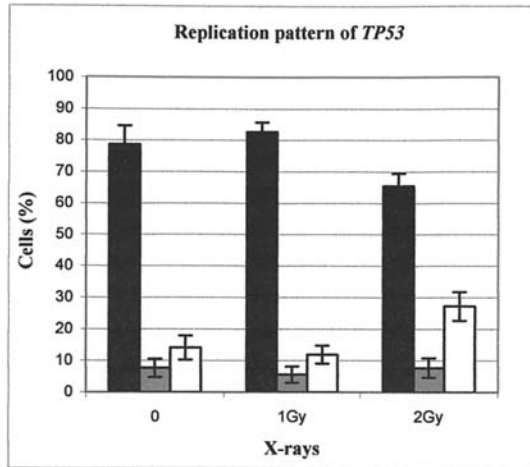


Figure 2. Replication pattern of the *TP53* locus in cells exposed to ionizing radiation (1 and 2 Gy). Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells after exposure to X-rays (2 Gy).

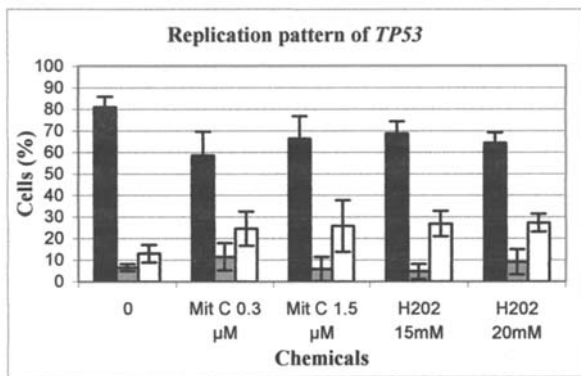


Figure 3. Replication pattern of the *TP53* locus in cells exposed to the chemicals mitomycin C (0.3 and 1.5 μ M) and H_2O_2 (15 and 20 mM). Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells after exposure to chemicals.

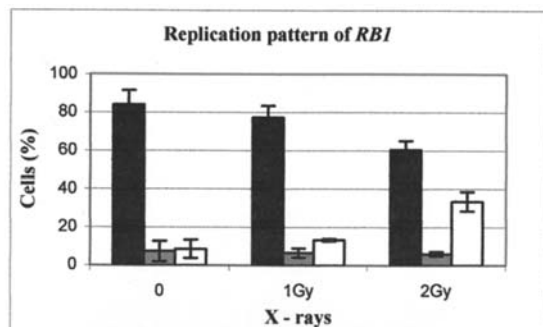


Figure 4. Replication pattern of the *RB1* locus in cells exposed to ionizing radiation (1 and 2 Gy). Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells after exposure to X-rays (1 and 2 Gy).

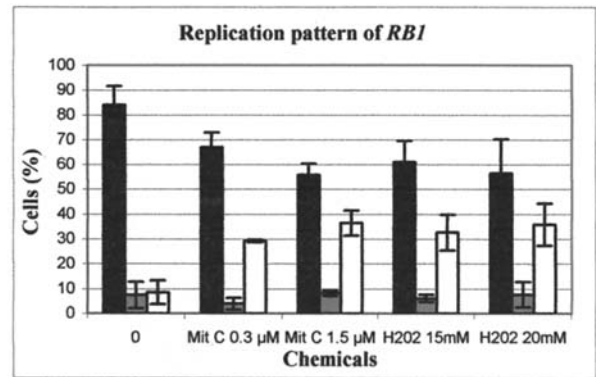


Figure 5. Replication pattern of the *RB1* locus in cells exposed to the chemicals mitomycin C (0.3 and 1.5 μ M) and H_2O_2 (15 and 20 mM). Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells after exposure to chemicals.

1.5 μ M, respectively (Fig. 3). Using the *RB1* probe, the frequency of the SS cells was 67 ± 8 and $56\pm 14\%$ and the frequency of the SD cells was 29 ± 7 and $36\pm 9\%$ after exposure to 0.3 and 1.5 μ M, respectively (Fig. 5).

H_2O_2 (15 and 20 mM) also led to an asynchrony of replication for both the *TP53* and *RB1* genes. Using the *TP53* probe, the frequency of the SS cells was 69 ± 6 and $64\pm 5\%$ and the frequency of the SD cells was 27 ± 6 and $27\pm 4\%$ after exposure to 15 and 20 mM, respectively (Fig. 3). Using the *RB1* probe, the frequency of the SS cells was 61 ± 8 and $57\pm 11\%$ and the frequency of the SD cells was 33 ± 7 and $36\pm 9\%$ after exposure to 15 and 20 mM, respectively (Fig. 5). The statistical analysis revealed a significant decrease of the SS cells and a significant increase of the SD cells in the treated cells ($P < 0.05$).

Globally, all the genotoxic agents used in the *in vitro* study, X-rays, H_2O_2 and mitomycin C produced a significant decrease of the SS nuclei and a significant increase of the SD nuclei when the two probes *TP53* and *RB1* were used (Table II and Figs. 2, 3, 4 and 5). Thus, genotoxic agents led to an asynchronous replication of DNA.

DNA replication timing in populations from Kazakhstan (in vivo study). The DNA replication timing assay by FISH was applied to the very same conditions to study human populations living in areas with different levels of pollution and displaying a high mortality rate from malignant tumours. While the population from Aktau (Mangistau area, Kora lake) had a high mortality from oesophagus and colon cancer and leukaemia, the population from the Ust-Kamenogorsk area had a high mortality from malignant tumours of several different organs (Table I). The study groups from Almaty, Aktau and Ust-Kamenogorsk were matched for age group, gender and smoking habits, with no significant differences (χ^2 test).

The frequency of the SS cells for *TP53* and *RB1* in peripheral blood lymphocytes in the control population (Almaty area) was 82 ± 7 and $79\pm 7\%$, respectively. For *TP53*, the frequency of the SD cells and DD cells was 10 ± 4 and

Table II. Replication pattern of the *TP53* and *RBI* loci in human lymphocytes exposed *in vitro* to ionizing radiation (1 and 2 Gy), chemicals (mitomycin C and H₂O₂) and controls.

Dose	<i>TP53</i>			<i>RBI</i>		
	SS (%)	DD (%)	SD (%)	SS (%)	DD (%)	SD (%)
0	81±6	7±2	13±4	84±8	7±5	9±5
1 Gy	83±3	6±3	12±3	77±6	7±2	13±1
2 Gy	65±4	8±3	27±5	61±5	6±1	34±5
Mit. C 0.3 μM	59±11	12±6	25±8	67±8	4±1	29±7
Mit. C 1.5 μM	66±10	6±6	26±12	56±14	8±5	36±9
H ₂ O ₂ 15 mM	69±6	5±4	27±6	61±8	6±3	33±7
H ₂ O ₂ 20 mM	64±5	9±6	27±4	57±11	8±3	36±9

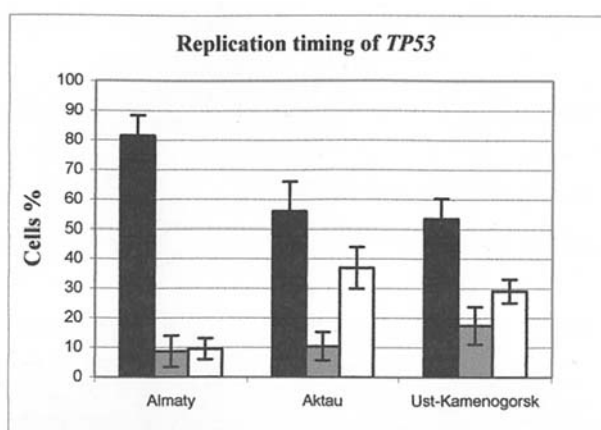


Figure 6. Replication timing of the *TP53* locus in the Kazakhstan populations, living in the Almaty (Chylik), Ust-Kamenogorsk and Aktau (Mangistau area, Kora Lake) areas. Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells, in the individuals living in the Ust-Kamenogorsk and Aktau areas (Mangistau area, Kora Lake) when compared with the controls from Almaty.

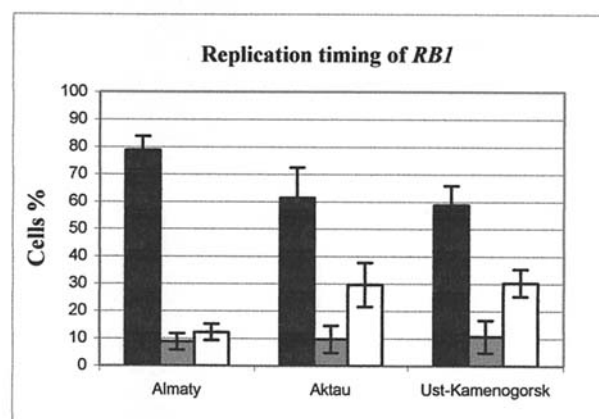


Figure 7. Replication timing of the *RBI* locus in the Kazakhstan populations, living in the Almaty (Chylik), Ust-Kamenogorsk and Aktau (Mangistau area, Kora Lake) areas. Each bar represents the mean of percentages of the SS, DD and SD nuclei. Black, SS; grey, DD; white, SD. Error bars represent standard deviation. The statistical analysis indicates a significant decrease of the SS cells and a significant increase of the SD cells, in the individuals living in the Ust-Kamenogorsk and Aktau areas (Mangistau area, Kora Lake) when compared with the controls from Almaty.

9±5%, respectively. For *RBI*, the frequency of SD and DD cells was 12±3 and 9±4. These data indicate a synchrony of replication in the control population.

In contrast, in individuals from Aktau (Mangistau area, Kora lake) and the Ust-Kamenogorsk areas a decreased number of SS cells and a higher number of SD cells were detected when the replication timing of both the *TP53* and *RBI* genes were studied as compared to the controls from Almaty ($P<0.001$, Table III and Figs. 6 and 7). The SS frequencies decreased to 56±10 and 61±11% in the Aktau area and 53±7 and 59±7% in the Ust-Kamenogorsk area. The SD frequencies increased to 37±7 and 30±8% in Aktau and 29±4 and 30±5% in the Ust-Kamenogorsk area. The frequency of DD cells in Ust-Kamenogorsk also increased as compared with the control area (17±6 vs. 9±5%) for the *TP53* gene ($P<0.001$, Fig. 6).

Taken together these results suggest that individuals living in polluted areas have a higher DNA replication asynchrony when compared with the control population living in Almaty. Additionally, when the population of Ust-Kamenogorsk, a

polluted area with a higher cancer mortality rate than Aktau, was compared with this one, a statistically significant difference in DNA replication patterns for *TP53* and *D22S163* loci was observed ($P<0.005$, Table III).

Although a difference in the SS and SD patterns was noted between the Almaty and Aktau populations when the *D22S163* locus was studied ($P<0.001$), we could not detect any statistically significant difference between Almaty and Ust-Kamenogorsk using the same probe (Table III).

Additionally, when the replication timing of the three loci was compared, the *D22S163* locus significantly differed from both the *TP53* and *RBI* genes ($P<0.002$).

Discussion

A wide range of environmental genotoxic agents can react with DNA or stimulate the production of reactive oxygen species, leading to DNA damage. DNA lesions may alter the conformational properties of strands at the replication fork,

Table III. Replication pattern of *TP53*, *RBI* and *D22S163* loci in lymphocytes from the Kazakhstan populations, living in the Almaty (Chylik), Ust-Kamenogorsk and Aktau areas (Mangistau area, Kora Lake).

Area	Locus	SS (%)	DD (%)	SD (%)
Almaty (Control)	<i>TP53</i>	82±7	9±5	10±4
	<i>RBI</i>	79±7	9±4	12±3
	<i>D22S163</i>	91±5	4±3	5±3
Aktau	<i>TP53</i>	56±10	7±5	37±7
	<i>RBI</i>	61±11	10±5	30±8
	<i>D22S163</i>	82±3	7±3	11±3
Ust-Kamenogorsk	<i>TP53</i>	53±7	17±6	29±4
	<i>RBI</i>	59±7	11±6	30±5
	<i>D22S163</i>	92±8	2±3	5±5

thus affecting the selectivity of polymerases and the fidelity of transcriptional machinery (19). DNA lesions are also triggering events of chromosomal aberrations. Chromosome translocations result in the generation of delays in replication timing/delay in the mitotic chromosome condensation phenotype. This phenotype occurs frequently in tumour cells and in cells exposed to ionising radiation both *in vitro* and *in vivo* (4,5). Other chromosome alterations such as HSRs are early replicating (4).

A switch to early replication precedes or coincides with the transcriptional activation of genes within the affected domain (20). Using genomic clone DNA microarrays to assess the replication timing of sequences in human genome during the S phase of the cell cycle, Woodfine *et al* (13) show a positive correlation between replication timing and transcriptional activity. Moreover, the DNA replication-timing analysis of the human chromosome 22 revealed that expressed sequences are replicated earlier than non-transcribed regions (21).

The data obtained in the present study, show that known DNA-damaging agents, e.g. H₂O₂, ionising radiation and mitomycin C led to an asynchronous replication timing of the genes *TP53* and *RBI*. This is demonstrated by the significant decrease of the SS cells and the significant increase of the SD cells (Table II and Figs. 2, 3, 4 and 5).

These data may be explained by the exposure to genotoxic agents which leads to a large spectrum of DNA and chromosomal lesions, some of which give rise to aberrations in the replication timing in the site of the lesions. The positive correlation between dicentric formation, the specific lesion of radiation and the frequency of SD cells found, suggests that DNA and chromosomal damage may be associated with asynchronous DNA replication.

Moreover, agents that introduce lesions in DNA could transiently inhibit DNA synthesis. This inhibition results in an altered replication control documented by the increased number of initiations of DNA synthesis (reviewed in ref. 22).

An asynchronous replication of the *TP53* and *RBI* genes in association with their activation cannot be ruled out. Both p53 and RB are two main tumour-suppressor pathways that

control cellular responses to potentially oncogenic stimuli (reviewed in ref. 23). It was reported that p53 is usually moderately expressed in normal cells and becomes active under stressful conditions (24) and can be activated by a wide spectrum of DNA-damaging agents. Its induction is an indicator of genotoxic damage (25). Islaih *et al* (26) exposed the human TK6 lymphoblastoid cell line to genotoxicants that cause genotoxic stress by many different mechanisms, including covalent modification and reactive oxygen species and found an activation of the p53 pathway in all the compounds. Comparing gene-expression profiles measured in human cells stably knocked down for ATM, NF-κB and p53, Elkon *et al* observed that NF-κB and p53 mediated most of the damage-induced gene activation (27).

One of the major roles of p53 in stress response is that of a sequence-specific transcription factor and *RBI* is a p53 effector (28).

The populations from the Ust-Kamenogorsk area and Aktau (Mangistau area, Kora lake) are seemingly exposed to genotoxic agents as a consequence of pollutant exposure. The former has an important mortality from malignant tumours of different organs and the latter has a high mortality from oesophagus and colon cancer and leukaemia as compared with the control region Almaty (Table I).

In individuals from these two areas an increase of the SD cells and a decrease of the SS cells was detected for the *TP53* and *RBI* genes as compared to the controls (Table III and Figs. 6 and 7). This behaviour is similar to that observed in human lymphocytes exposed *in vitro* to known genotoxic agents. In individuals from the Ust-Kamenogorsk area the percentage of cells where the two *TP53* alleles are both replicated (DD cells) is significantly higher than in the controls from Almaty, indicating a switch to early S phase.

On the other hand, given the significant difference in the SS and SD patterns noted between the Almaty and Aktau populations as well as in the SS, SD and DD patterns between Aktau and Ust-Kamenogorsk, when the *D22S163* locus was studied, we could not rule out that these abnormalities in the DNA replication timing may contribute to put into evidence

an overall, non-locus-specific event associated with genetic instability. This event seems not to exclude the association between DNA lesions, activity of *TP53* and *RBI* and replication asynchrony, because the replication pattern of the *D22 S163* locus significantly differs from those of the *TP53* and *RBI* loci.

Although asynchronous replication is characteristic of monoallelic expression in imprinted genes and is therefore an epigenetic mark (29), the data presented seem to indicate that DNA lesions may result in the asynchrony of DNA replication.

Taken together the present data suggest that asynchronous DNA replication as detected by FISH may be a potentially useful and predictive approach in estimating exposure to genotoxic/carcinogenic agents. The application of this methodology in larger populations could give rise to an important tool for the identification of highly-exposed individuals at the population level, allowing for public health strategies in order to reduce cancer mortality.

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