Changes in DNA Capacity for Actinomycin-D Binding in Nuclei of Interphase Cells

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Abstract. ^{3}H-AMD binding to DNA in interphase nuclei was tested on asynchronous and synchronous LS/BL cell populations under physiological conditions and after exposure to gamma rays (^{60}Co). ^{3}H-AMD binding to DNA in an asynchronous cell population appeared to be nearly constant and independent of ^{3}H-AMD concentration. However, in comparing individual cells, a great variability could be observed. In synchronized cells the DNA accessibility for ^{3}H-AMD binding changed in the course of the cell cycle, with a maximum occurring at the late G_1-phase (13.95×10^{-12} μmol/nucleus) and a minimum at the late G_2-phase (2.63×10^{-12} μmol/nucleus). In irradiated cells the DNA capacity for ^{3}H-AMD binding was growing with the increasing dose (5—80 Gy) from 4.9 to 11.2×10^{-12} μmol ^{3}H-AMD/nucleus.

Key words: ^{3}H-AMD-DNA interaction — Cell cycle — Gamma rays

Introduction

In proliferating cells the chromosomes go through a cycle of replication, condensation, anaphase separation, decondensation and subsequent reassembly into daughter nuclei. Despite a good understanding of the synthesis of macromolecular components of chromatin during the cell cycle of eukaryotic cells, information about the status of chromosomes throughout the interphase is limited. The data reviewed (e.g. Rao and Johnson 1974) indicate that during a cell cycle the chromosomes pass through a continuum of conformational changes, with the two extremes, mitotic and S-phase chromatin, reflecting the greatest and the least degrees of condensation, respectively. However, changes in the grain distribution in autoradiographs of HeLa cells nuclei labelled with ^{3}H-dTh at different time intervals of the S-phase implied chromatin condensation in the course of the S-phase (Beneš and Rotreklová 1977).

The rather subtle changes taking place in the organization of chromatin may be shown by means of the binding of actinomycin-D (AMD) to DNA in interphase nucleus (Ringeretz et al. 1969; Berlowitz et al. 1969). The binding sites are

accessible deoxyguanosine residues, with the highest affinity to d(G/C) sequences (Sobell et al. 1971).

In experiments described below, ^{3}H-AMD binding to DNA in the nuclei of asynchronous and synchronous LS/BL cell populations, and in LS/BL cells exposed to gamma rays was tested. ^{3}H-AMD binding to DNA in asynchronous cell populations appeared to be nearly constant, while in synchronous cells significant changes (with a maximum at the late G_1-phase, and a minimum at the late G_2-phase) were observed. In irradiated cells ^{3}H-AMD binding to DNA was growing with the increasing dose of irradiation.

Materials and Methods

Mouse lymphosarcoma cells (LS/BL) were used. The cells were propagated in the peritoneal cavity of female C57 black/10 mice. Cell suspension, containing 10^7 cells/ml, was prepared 5 days after inoculation in TC medium L15 (Difco Laboratories) and inactivated bovine serum (2:1; Bioveta). Experiments were carried out in vitro. The duration of cell cycle phases was estimated by Velčovský et al. (1982) according to the method of Lajtha et al. (1954).

The cells were labelled in a medium supplemented with 74 kBq ^{3}H-AMD (Radiochemical Centre Amersham; specific activity 188 GBq/mmol). The original ^{3}H-AMD was diluted with cold Dactinomycin (Lyovac Cosmegen) to give a final specific activity of 1.85 GBq/mmol. Free ^{3}H-AMD was removed by fivefold washing of the cells in cooled 95 % ethanol.

The ^{3}H-activity of DNA was detected by means of high-resolution autoradiography (standard procedure using stripping film Kodak AR.10) or using a beta-spectrometer Nuclear Chicago Mark 2, after cell solubilization in an NCS solubilizer (Nuclear Chicago).

The cells were irradiated with gamma rays using a ^{60}Co-unit (Chirana Prague) at room temperature and a dose rate of 4.1 Gy/min.

A double block with 7.5 mmol dTh was used for cell synchronization. The 12h-blocks were separated by a 4 h growing period in a fresh medium. The dTh block was reverted with 1.0 mmol dC. The cell viability was estimated by staining with 0.1 % eosin. Dead cells were removed during 15 min incubation with 0.1 % trypsin.

Results

Asynchronous cell population. ^{3}H-AMD binding to DNA was tested on living cells. In the first group of experiments ^{3}H-AMD with original specific activity (188 GBq/mmol) was used. ^{3}H-AMD penetrated quickly into the nucleus and was bound to DNA (Fig. 1). A prolongation of the labelling time to more than 30 min did not result in any further increase in the DNA ^{3}H-activity.

In order to rule out possible exhaustion of free ^{3}H-AMD and subsequent limited DNA labelling, the original specific activity of ^{3}H-AMD was lowered to 1.85 GBq/mmol using cold Dactinomycin. The cell suspension was divided into three groups and incubated with 4.0×10^{-2} μmol (74.0 kBq/ml), 12.0×10^{-2} μmol (222.0 kBq/ml), and 24.0×10^{-2} μmol (444.0 kBq/ml) ^{3}H-AMD, respectively during 30 min. The resulting ^{3}H-AMD activity bound to DNA was similar in all

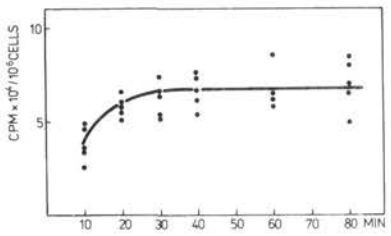

Fig. 1. ^{3}H-AMD binding to DNA as a function of labelling time.

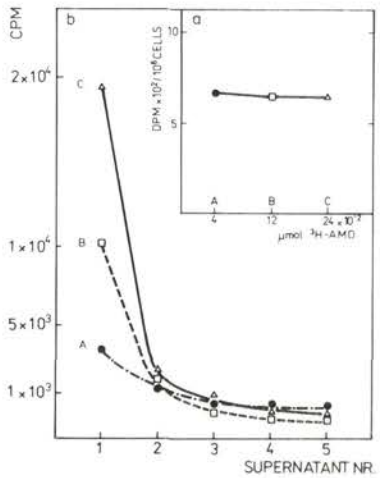

Fig. 2. Labelling of the cells with different ^{3}H-AMD concentrations: *a* — ^{3}H-AMD bound to DNA; *b* — free ^{3}H-AMD in supernatants after fivefold washing of cells.

experimental groups (Fig. 2*a*), i.e. it was independent of the ^{3}H-AMD concentration applied. The validity of the result was checked by estimating free ^{3}H-AMD activity in the solutions used for cell washing (Fig. 2*b*). The free ^{3}H-AMD activity was proportional to the activity added to the medium, and the procedure used was sufficient to remove it.

In the asynchronous cell population the average DNA capacity for ^{3}H-AMD binding was found to be constant. On the other hand a detailed analysis of cell labelling in autoradiographs revealed great differences in DNA binding capacity between individual cells (Fig. 3).

Labelled cells were smeared, dried at room temperature, washed in 95 % ethanol and coated with stripping film Kodak AR.10. After 240 h of exposure the grain counts per 1000 labelled nuclei were checked. With respect to the grain

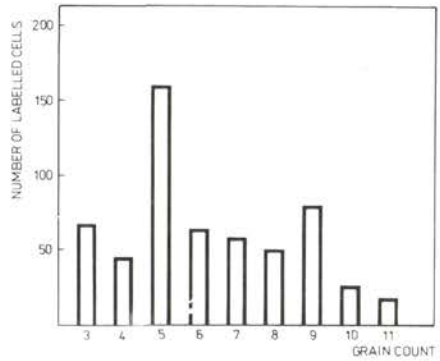

Fig. 3. Variability in the ^{3}H-AMD binding to DNA in an asynchronous cell population. Frequency of cells labelled with the given graincounts per nucleus in autoradiographs.

distribution in the background and the area of a nucleus, 3 grains per nucleus were demanded in order to evaluate a cell as being labelled (Hill and Beneš 1966). The number of labelled cells amounted to 55.3 %. Grain counts per one labelled cell ranged between 3 and 11 grains. This experiment suggested that the cells were synchronized.

Synchronous cell population. After the completion of cell synchronization (the synchrony degree, estimated on the basis of ^{3}H-dTh incorporation into DNA as detected by means of autoradiography, was about 92—95 %, for the S-phase as a whole), the cells were resuspended in a fresh medium, divided into 2 ml aliquots and incubated for 30 min with 74.0 kBq ^{3}H-AMD/ml. Following time intervals were chosen: -2; -1; 0; 1; 2; 3; 4; 5; 6; and 7 h. Interval 0 indicated the start of the S-phase. At the given time intervals, ^{3}H-AMD was added to the cell suspension; thus data summarized in Fig. 4 represent DNA binding capacity at $t_n + 30$ min. Fig. 4 shows a correlation of DNA capacity for ^{3}H-AMD binding with the timetable of the LS/BL cell cycle. In the late G_1 — phase (peak) 13.95×10^{-12} μmol ^{3}H-AMD was bound to DNA in a cell nucleus. During the 1st hour of S-phase, the DNA binding capacity was rapidly decreasing and remained nearly constant thereafter, amounting to 4.27×10^{-12} μmol ^{3}H-AMD/nucleus. The passage of cells into G_2 — phase was associated with a further decrease in DNA binding capacity to 2.63×10^{-12} μmol ^{3}H-AMD/nucleus.

Irradiated cell population. A suspension of the asynchronous cell population was divided into 2 ml aliquots placed in plastic test tubes and exposed to 5; 10; 20; 40; and 80 Gy gamma rays (^{60}Co). The tubes were irradiated simultaneously and

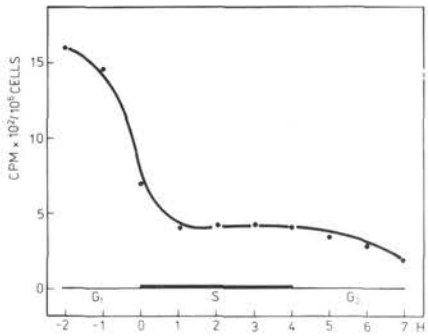

Fig. 4. Changes in DNA capacity for ^{3}H-AMD binding in the course of the cell cycle.

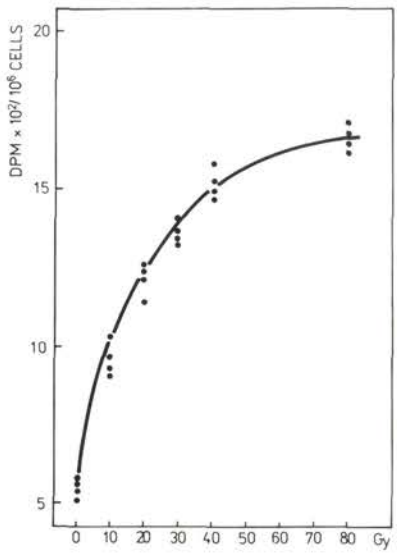

Fig. 5. DNA capacity for ^{3}H-AMD binding as a function of the dose of gamma irradiation.

samples were taken following the exposure to given doses. For this reason only 5 Gy were a single dose, the higher doses being fractionated. The interval between the irradiations was about 2 min. Immediately after the irradiation the cells were incubated for 30 min with 74 kBq ^{3}H-AMD/ml. The DNA capacity for ^{3}H-AMD binding increased with the dose of radiation (Fig. 5). In addition, the effect of the time interval from the end of irradiation (20 Gy) to ^{3}H-AMD addition was observed. Intervals of 0; 1; 2; 3; 4; 5; and 6 h were studied. The DNA capacity for ^{3}H-AMD binding was growing for 5 h after the irradiation (Fig. 6).

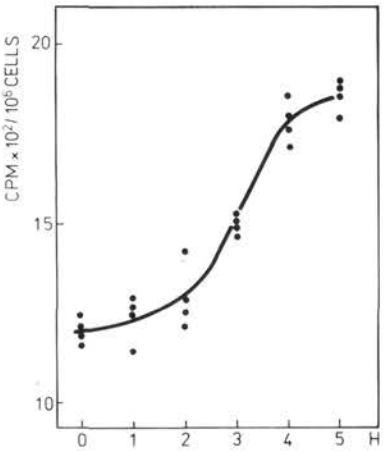

Fig. 6. DNA capacity for ^{3}H-AMD binding as a function of the time after exposure to 20 Gy.

Treatment of cells with 0.2 mol/l sulphuric acid. A cell suspension prepared by standard procedure was centrifuged and the cells were resuspended for 20 min in 0.2 mol/l sulphuric acid. The cells were subsequently labelled for 30 min with 74 kBq ^{3}H-AMD per ml. Table 1 shows an increase in DNA capacity for ^{3}H-AMD binding after partial histone extraction as compared with nonirradiated and 10 Gy-irradiated cells.

Discussion

The three-dimensional structure of the crystalline complex containing AMD and deoxyguanosine has shed light on the stereochemistry of AMD-binding to DNA. The phenoxazone ring system of AMD intercalates into DNA helix, while deoxyguanosine residues interact with both cyclic peptides through specific hydrogen bonds (Sobell et al. 1971). The conformation of histones plays a very important role in chromosome structure (Bradbury et al. 1973). The AMD-DNP interactions require accessible sections of the DNA molecule to be either weakly bound to the histone or "naked". Gaps of histone-free DNA between two histone-bound segments were shown on the basis of melting temperature of native chromatin (Li 1975).

During 20 min treatment of cells with 0.2 mol/l sulphuric acid about 20 to 40 % histones were removed. The histone extractability was cell cycle phase-dependent (Beneš and Rotreklová 1974). The histone removal was accompanied with an increase in DNA capacity for ^{3}H-AMD binding (Table 1). The disproportion

Table 1. Effect of experimental procedures on DNA capacity for ^{3}H-AMD binding

Key	LS/BL cells		
	nonirradiated	irradiated 10 Gy	treatment with 0.2 mol/l H_2SO_4
DPM	536.8	822.0	2 959.5
	743.6	938.0	2 055.8
	527.8	963.5	3 867.2
	630.0	568.9	2 955.5
Mean DPM	633.8	823.1	2 959.5
Cell counts	930 000.0	840 000.0	760 000.0
DPM/10^6 cells	681.0	979.0	3 894.0
%	100.0	143.7	571.8

between the amount of histones lost (40 %) and the increase in ^{3}H-AMD bound to DNA (570 %) suggested that it was the dissociation of DNA-histone bonds that played an important role in AMD-chromatin interactions. For this reason great attention should be paid to ionic strength of the solutions used (Bolund 1970), and acid fixatives (Brody 1974). Taking into account the factors mentioned, our experiments were carried out using standard procedures and observations were made on living cells.

In the asynchronous population of LS/BL cells under the above conditions the average DNA capacity for ^{3}H-AMD binding appeared to be nearly constant. As this could reflect an exhaustion of the available ^{3}H-AMD, its specific activity was lowered 100× using cold AMD. The results of this group of experiments (Figs. 2*a*, *b*) corresponded well to the previous one (Fig. 1). Maybe this is why AMD binding to DNA has been supposed to be a relative measure of the non-repressed part of the genome (Ringeretz and Bolund 1969; Brachet and Hulin 1970). However, the histogram (Fig. 3) suggests a great variability in ^{3}H-AMD binding to DNA between single cells. The actual variability was greater due to the fact that only cells with at least 3 grains/nucleus were taken into account. For this reason the labelling index amounted to 55.3 % only.

Experiments on synchronized cells showed changes in DNA accessibility for ^{3}H-AMD binding in the course of the cell cycle, i.e. chromatin conformational changes. The highest DNA accessibility for ^{3}H-AMD binding was observed in the late G_1- and early S-phases. These findings are in good agreement with those in HeLa cells (Pederson and Robbins 1972), except for the rate of the decrease

during both the S- and G_2-phases. In HeLa cells the DNA binding capacity for ^{3}H-AMD was decreasing slowly throughout both the S- and G_2-phases, while in LS/BL cells it was dropping during the 1st hour after the start of the S-phase with no further significant changes. A slow decrease followed after the cells had entered the G_2-phase, with a minimum occurring at the completion on of this phase. The opinion that S-phase cells exhibit the lowest degree of chromatin condensation (Rao and Johnson 1974) does not hold for S-phase as a whole, but only for its initial stage.

Radiobiological literature reports on findings which allow the presumption of an increase in the DNA capacity for AMD binding in irradiated cells, e.g., an increase in DNA sensitivity to acid hydrolysis in irradiated cells due to the dissociation of DNA-histone bounds and histone loss (Beneš and Rotreklová 1966). This presumption was experimentally proved in the present experiments on LS/BL cells.

Immediately after the irradiation the increase in ^{3}H-AMD bound to DNA was significant from 5 to 80 Gy gamma rays (^{60}Co). Below 5 Gy and above 80 Gy changes were statistically insignificant. The average DNA binding capacity was growing with the radiation dose from 4.9 to 11.2×10^{-12} μmol ^{3}H-AMD/nucleus. On the other hand, the physiological variability of ^{3}H-AMD bound to DNA ranged from 2.63 to 13.95×10^{-12} μmol/nucleus. However, physiological changes in the chromatin structure were reversible, while the radiation damage to the chromatin was irreversible during the interval of observation and developed with the time after the irradiation. Early conformational changes in the chromatin of the interphase nuclei were detected by ^{3}H-AMD binding to DNA in irradiated cells.

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