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THE FORMATION OF HIGH MOLECULAR WEIGHT DNA FRAGMENTS: THE HALLMARK OF APOPTOSIS OR EARLY GENOMIC EVENTS IN STRESS RESPONSE?

We studied the peculiarities of nuclear DNA fragmentation in primary culture of murine thymocytes and in human lymphoblastoid cell culture (line CEM) induced to apoptosis by various influences. We showed that in apoptotic cells the ordered high molecular weight DNA cleavage, recognizable as DNA fragment release of 50—100 and 250—300 kb in length, preceded to typical nuclear DNA fragmentation at the internucleosomal regions. Comparative study of the high molecular weight and internucleosomal DNA fragmentation demonstrated that these show different sensitivity to Zn^{2+} ions, actinomycin D and cycloheximide. In addition, the formation of large molecular weight fragments in contrast to oligonucleosomal ones may be reverted in conditions promoting to topoisomerase II mediated rejoining of cleaved DNA. These finding suggest that the high molecular weight and internucleosomal fragmentation may follow two different programmes of DNA cleavage during apoptosis mediated, presumably, by diverse enzymes.

We showed that similar processes of ordered high molecular weight DNA cleavage occur in apoptotic cells and in those subjected to the stress challenges. The formation of high molecular weight DNA fragments was demonstrated to proceed promptly, show no correlation with cell death and occassionally may be of transient nature. The data obtained allow to interpret the formation of high molecular weight fragments as a component of cell response to stress rather that an early step of DNA fragmentation during apoptosis.

Introduction. Apoptosis (a programmed cell death) provides a widely spread physiological process, commonly occurring during embryogenesis in established tissues and upon various pathological conditions [1—3]. It possesses distinctive morphological features. One of the most documented biochemical characteristic of apoptosis is the internucleosomal DNA fragmentation to yield oligonucleosomal fragments which upon resolution by conventional gel electrophoresis develop typical «ladder» [4—6]. Lately there is growing evidence to suggest another type of ordered DNA cleavage during apoptosis resulting in appearance of high molecular weight (HMW) fragments of about 30—500 kb, which either precede to or are observed in the absence of internucleosomal fragmentation [7—9]. This finding provided evidence to suggest that the HMW-DNA fragmentation rather then internucleosomal one may be a key characteristic of apoptosis [9, 10].

In this report we present evidence to show that the ordered nuclear DNA cleavage into the HMW fragments seems to differ by its characteristics from internucleosomal fragmentation and occur not only during apoptosis, but in the cell subjected to the stress challenges.

Materials and Methods. Cell lines and culture conditions. In this work we used the human lymphoblastoma cultured cells (line CEM) and primary cell culture of murine thymocytes obtained from the thymus of a 4—5 week old mice (line BALB/c). CEM cultured cells were routinely incubated in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS) in an atmosphere of 95 % air, 5 % CO_2 to give a final suspension of 210 cell/ml. Thymocyte primary culture was prepared from intact thymocytes to final suspension of $2 \cdot 10^7$ cells in RPMI

1640/10 % FCS and incubated under conditions indicated above for at least 6 h either with or without apoptotic inducers. Apoptosis was induced by incubation of cells either with 1 μM dexamethasone or 7.5 μM teniposide (VM 26). The cell viability was estimated by Trypan blue exclusion.

Sample preparation. 200 μl of cell suspension were placed into the well of cell culture plate followed by addition of equal volume of 1 % low-melting agarose prepared on TEN-buffer (10 mM Tris-HCl, pH 1.5, 1 mM EDTA, 150 mM NaCl). After gelation the equal volume of lysing buffer (TEN+1 % SDS) was layered followed by incubation for 1 h at 37 °C. Agarose plugs containing the lysed cells were used for analysis by agarose gel electrophoresis.

Gel electrophoresis. Lysed cell preparations were fractionated either conventional or field inversion gel electrophoresis (FIGE) to detect the pattern of nuclear DNA cleavage. Conventional gel electrophoresis was carried out in 1.4 % agarose at 50 V for 4—5 h using 0.5× ×TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8—8.5). FIGE was performed in 1 % agarose at 85 V for 18 h in 0.5×TBE buffer under constant pulses of electric field (24 s «forward» and 8 s «backward») allowing to monotonous resolution of DNA molecules sized up to 500 kb [11]. In some cases FIGE was carried out for 5—6 h allowing to resolve both low- and HMW DNA. After electrophoresis the gel was stained with 1 mg/ml ethidium bromide for 10 min, viewed using a UV transilluminator and photographed using Mikrat 300 film.

Results and Discussion. Previously we showed that the treatment of agarose-embedded nuclear DNA preparations with the protein denaturants resulted in appearance of two main types of nuclear DNA fragments sized about 50—100 and 250—300 kb, which proved to be unityped for various eukaryote representatives [12]. The ordered cleavage of nuclear DNA into the HMW fragments (HMW-fragmentation) seems to be of physiological value since the formation of HMW DNA fragments may be involved in the early events accompanying the programmed cell death. This is evidenced from the data presented in Fig. 1, which show that the dexamethasone induced apoptosis in primary culture of thymocytes is associated with extensive nuclear HMW-DNA cleavage, which precedes to typical for apoptosis internucleosomal DNA fragmentation and is detectable immediately after cell treatment with dexamethasone. In the absence of dexamethasone certain proportion of cells seems to undergo the spontaneous apoptosis accompanied with the HMW- and internucleosomal fragmentation (Fig. 1).

Incubation of apoptotic thymocytes with Zn^{2+} ions, which are known to inhibit the apoptosis specific endonucleases, demonstrates that these completely counteract to the internucleosomal DNA fragmentation but fail to prevent the accumulation of HMW DNA fragments (Fig. 1). Moreover, Zn^{2+} ions seem to increase the dexamethasone induced HMW-DNA cleavage at least at the early stage of apoptosis (Fig. 1).

The fact that the internucleosomal and HMW-DNA fragmentation show various response to Zn^{2+} ions suggests that they may follow two different, with regards to their properties, programmes of DNA cleavage during apoptosis. It is supported by the observation that the internucleosomal DNA fragmentation in contrast to the HMW-DNA cleavage is completely prevented by actinomycin D and cycloheximide (results not shown).

In addition, the formation of HMW DNA fragments but not the internucleosomal DNA fragmentation may be reversible. This, however, is to be the case only at the early steps of apoptosis (Fig. 2).

Similarly to the primary culture of thymocytes, in cultured human lymphoblastoma cells (line CEM) the apoptosis to be provoked either by dexamethasone or teniposide is accompanied with progressive DNA cleavage into HMW-DNA fragments of about 250—300 and 50—100 kb with the intensity of fragmentation being correlated with cell death

(Fig. 3). However, in control cells (cells non stimulated to apoptosis) the HMW-DNA cleavage is also observed without any relationship with changes in cell viability (Fig. 3). The data presented suggest that the formation of HMW-DNA fragments does not correlates with the cell death directly and may be observed in cells non stimulated to apoptosis.

As evidenced from the data presented in Fig. 4 the recovery of cells after heat shock is accompanied with the transient increase in HMW-

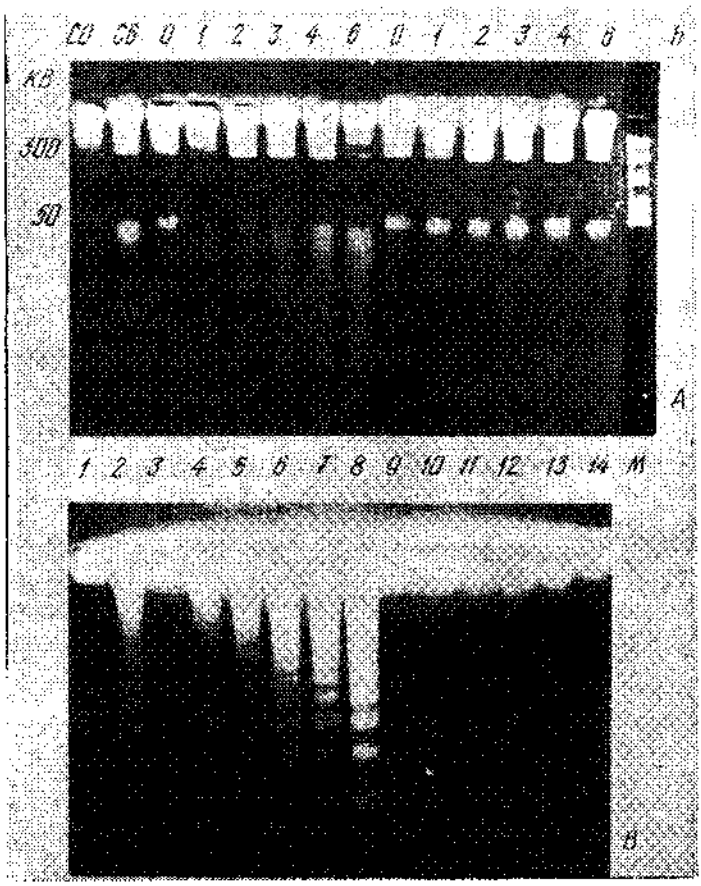


Fig. 1. Gel electrophoretic analysis of DNA degradation during dexamethasone induced apoptosis in thymocytes. Cells were treated with 1 μM dexamethasone and allowed to incubate for the time designated at the top of the Figure (h) without (lanes *1—8*) or with 1 mM Zn^{2+} (lanes 9—14). After incubation cells were embedded into low-melting agarose, treated with SDS (see Materials and Methods) and fractionated either by FIGE (*A*) or conventional gel electrophoresis (*B*). *C0* — control cells (not treated with dexamethasone) at the beginning of *in vitro* incubation, *C6* — the same cells after 6 h *in vitro* incubation, *M* — lambda oligomers. Time of incubation designated on the top of the figure, numbers of lanes designated between the panels *A* and *B*, these designations are shared by both panels of the figure

DNA cleavage which is observed after 2 h of cell incubation at normal temperature and seems to disappear after 4 h incubation. Teniposide, the specific modulator of topoisomerase II activity, was shown to accelerate the transient changes in the integrity of HMW-DNA domains (Fig. 4). Similarly, cell incubation in serum deficient medium is also accompanied with the increase in HMW-DNA fragmentation which rapidly declines following serum addition and after 24 h incubation with fresh serum disappears (Fig. 5). These data suggest that the formation of HMW-DNA fragments takes place in cells subjected to the stress challenges and is reversible after removal of stress influences.

Thus, the data presented indicate that the formation of HMW-DNA fragments occurs at the early stages of apoptosis and seems to differ from the internucleosomal fragmentation with regards to its properties. The HMW-DNA cleavage may be detected immediately after apoptosis induction, takes place in normal cells and in those subjected to stress challenges, may not correlate with cell viability and seems to be reversible. This allows to interpret the formation of HMW-DNA fragments

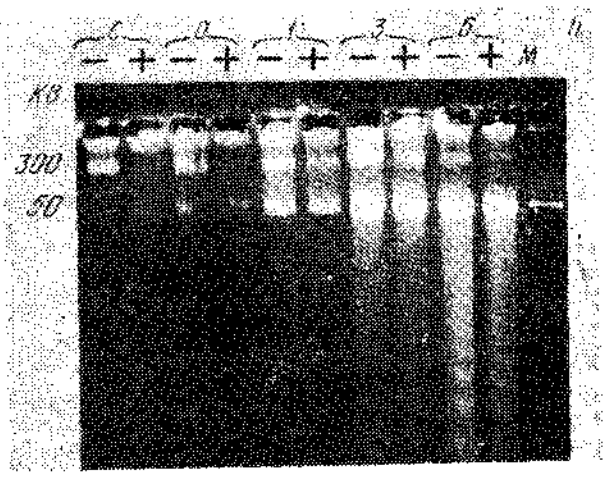
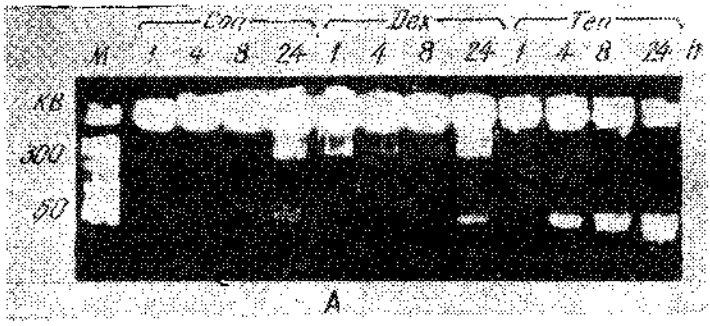


Fig. 2. The rejoining of cleaved high molecular weight DNA fragments in apoptotic thymocytes. Cells were treated with 1 μM dexamethasone and allowed to incubate for the time designated at the top of the Figure (h). After incubation cells were embedded into agarose (—) or incubated at 55 °C for 10 min (+) followed by agarose embedding. After gelation cells were treated with SDS and fractionated by FIGE under conditions allowing to separate both large- and low molecular weight fragments; *C* — control cells (not treated with dexamethasone), *M* — lambda DNA



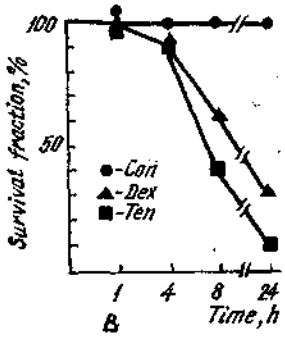


Fig. 3. The ordered DNA cleavage during apoptosis in cultured CEM cells: *A* — Cells were incubated for the time designated at the top of the Figure (h) without apoptosis inducers (Con) or with 1 μM dexamethasone (Dex) or with 7.5 μM teniposide (Ten) (After incubation cells were embedded into agarose, treated with SDS and fractionated by FIGE; *M* — lambda oligomers); *B* — The time course of cell viability estimated by Trypan blue exclusion

as being not a hallmark of apoptosis but rather as a reaction of cell genome to stress challenges.

Our results as well as the data reported earlier [7—10] demonstrate that the process of programmed cell death is accompanied with the ordered cleavage of nuclear DNA into HMW fragments, which precedes to the internucleosomal DNA fragmentation. At the same time our findings reveal that the HMW-DNA cleavage and internucleosomal DNA fragmentation seem to differ in regard to their characteristics and may present diverse processes of DNA cleavage during apoptosis. The fact that formation of HMW fragments and internucleosomal DNA cleavage

exhibit distinct sensitivity to Zn^{2+} ions, actinomycin D and cycloheximide suggests that different enzymes may be involved into these processes. The observation that topoisomerase II specific poison teniposide may modulate the heat-shock mediated turnover of HMW fragments (Fig. 4) suggests the topoisomerase II involvement in HMW-DNA cleavage. This

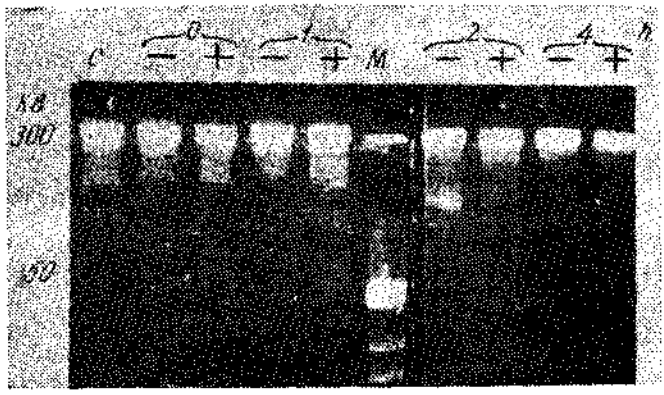


Fig. 4. The transient nuclear DNA cleavage in cultured CEM cells during heat-shock recovering. Cells were heat-shocked at 42 °C for 10 min followed by incubation at 37 °C for the time designated at the top of Figure (h) without (—) or with 7.5 μM teniposide (+). Following incubation cells were embedded into agarose, treated with SDS and fractionated by FIGE; *C* — control cells; *M* — molecular weight standards: lambda DNA+*HindIII* digest of lambda DNA

is further confirmed by the fact (Fig. 2) that the HMW-DNA fragmentation may be reversible in conditions that initiate topoisomerase II mediated DNA cleavage toward religation of cleaved DNA [13—15].

Our data show that the formation of HMW-DNA fragments takes place both in apoptotic cells and those subjected to stress challenges. Since any stress influences may interfere with cellular viability one should believe that the increased HMW-DNA cleavage may reflect the fact that

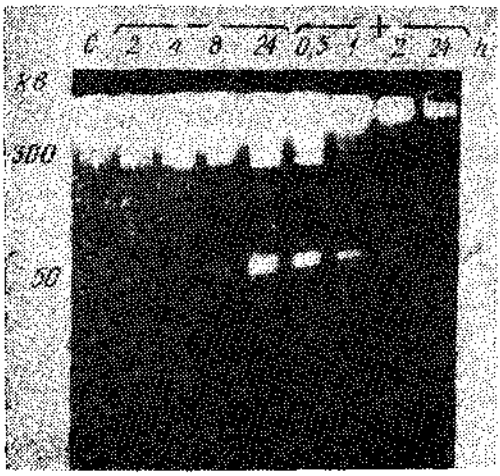


Fig. 5. The transient nuclear DNA cleavage in cultured CEM cells during serum starvation. Cells were incubated in serum-free medium (—) for the time indicated at the top of Figure (h). Following starvation serum was added cells were incubated in serum-containing medium (+) for the time indicated at the top of Figure (h). After incubation cells were embedded into agarose, treated with SDS and fractionated by FIGE; *C* — control cells before serum starvation

cells become predisposed to the apoptosis. However, our data show that formation of HMW-DNA fragments fails to correlate directly with the changes in cell viability, takes place in normal cells and is reversible after removal of stress influences. In addition, in apoptotic cells the increased HMW-DNA cleavage may be detected immediately after cell treatment with dexamethasone or Zn^{2+} ions (Fig. 1). These findings add credence to the idea that the formation of HMW-DNA fragments represents more likely the cellular response to stress than the early step of apoptotic nuclear DNA fragmentation. In this context it is of interest the observation that the loss of capacity to religation of the HMW-DNA

fragments at elevated temperature is coincident with oligonucleosomal ladder development during apoptosis (Fig. 2). This can be explained by the loss of topoisomerase II activity at this step of apoptosis due to non-specific proteolysis which may occur in apoptotic cells [16, 17]. Alternative explanation may be that at the early steps of apoptosis the formation of HMW-DNA fragments, mediated presumably by topoisomerase II, represents the cellular response to apoptotic inductors and may not be directly implicated in «apoptosis-specific» DNA fragmentation, which takes place at the advanced stages of apoptosis coinciding with the development of oligonucleosomal ladder.

Our interpretation of HMW-DNA cleavage is based on previously obtained data showing that fractionation of nonapoptotic lysed nuclear preparation by pulsed field gel electrophoresis results in appearance of subset of discrete DNA fragments correlating with the higher levels of chromatin structural organization [12]. In accordance with our early data as well as with those obtained by other workers the fragments of about 50—100 kb may be ascribed to nuclear DNA loop domains [12, 18, 19]. We provided evidence that nuclear DNA structural domains are involved in functioning DNA/topoisomerase complex with its ability to mediate the cleavage/religation equilibrium reactions [20].

Based on the studies with purified topo II enzymes and DNA the two-stage model has been proposed for topoisomerase II-mediated cleavage/religation reactions [13—15]. According to this model the enzyme/DNA cleavable complex is the key covalent intermediate in the topoisomerase II mediated DNA turnover, which is in rapid equilibrium with noncleavable complex. The exposure of the cleavable complex but not noncleavable one to protein denaturants (such as SDS or alkali) results in cleaved DNA product involving the covalent linking of topoisomerase II subunits to the 5′-ends of broken DNA [13—15].

Our and other data reveal that the decisive prerequisite for the HMW-DNA cleavage seems to be the exposition of agarose embedded nuclear and cellular preparations either to protein denaturants (SDS) or to topoisomerase II specific poisons followed by SDS treatment [12, 19, 20]. No DNA fragment releases into gel provided these agents lacking even if nuclei were destroyed with high concentrations of EDTA or Triton X-100 [12]. Base on the SDS-dependent HMW-DNA cleavage as well as on the previous data suggesting the involvement of DNA structural domain in functioning DNA/topoisomerase complex it seems reasonable to note that the formation of HMW-DNA fragments at the early stages of apoptosis and upon stress challenge should be by no means associated with the real DNA fragmentation. It is appropriate to suggest that in these cases the changes in the integrity [9] of DNA structural domains takes place. In terms of functioning DNA/topoisomerase complex the changes in integrity of DNA structural domains may be interpreted as their transition from noncleavable to cleavable state mediated by topoisomerase II. Whether the specific subset of structural domains is involved in these conversions or changes in the integrity of random set of structural domains takes place remains to be elucidated.

To summarize the foregoing account one may conclude that the changes in the integrity of nuclear DNA structural domains detectable as changes in the pattern of the SDS-dependent HMW-DNA cleavage occur at the early stages of apoptosis and upon stress influences. The changes in the integrity of DNA structural domains may be interpreted as their turnover between cleavable and noncleavable states mediated by topoisomerase II and may be ascribed to the early genomic response to various stress challenges.

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ФОРМУВАННЯ ВЕЛИКИХ ФРАГМЕНТІВ ДНК: КЛЮЧОВА ХАРАКТЕРИСТИКА АПОПТОЗУ ЧИ РАННЯ ГЕНОМНА ВІДПОВІДЬ НА СТРЕС?

Резюме

В роботі досліджено особливості фрагментації ядерної ДНК у первинній культурі тимоцитів мишей і в культурі лімфобластоїдних клітин людини (лінія CEM), індукованих до апоптозу різними чинниками. Крім того, наведено дані про те, що процес формування великих фрагментів за своїми характеристиками відрізняється від міжнуклеосомної фрагментації і спостерігається не лише при апоптозі, але і в клітинах, підданих дії стресу. Зміна нативності ядерної ДНК у відповідь на стрес відбувається швидко, не є пов'язаною з загибеллю клітин і в деяких випадках їй притаманний транзитний характер. Отримані результати дозволяють інтерпретувати формування великих фрагментів скоріше як компонент клітинної відповіді на стрес, а не як ключову властивість апоптозу.

REFERENCES

1. *Wyllie A. H., Kerr J. F. R., Currie A. R.* Cell death: the significance of apoptosis // Int. Rev. Cytol.— 1980.— **68**.— P. 251—305.
2. *Arends M. J., Wyllie A. H.* Apoptosis: mechanisms and roles in pathology // Int. Rev. Exp. Pathol.— 1991.— **32**.— P. 223—254.
3. *Williams G. T., Smith C. A., McCarthy N. J., Grimes E. A.* Apoptosis: final control point in cell biology // Trends Cell Biol.— 1992.— **2**.— P. 263—267.
4. *Cohen J. J., Duke R. C., Fadok V. A., Sellins K. S.* Apoptosis and programmed cell death in immunity // Annu. Rev. Immunol.— 1992.— **10**.— P. 267—293.
5. *Wyllie A. H.* Glycocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation // Nature.— 1980.— **284**.— P. 555—556.
6. *Compton M. M.* A biochemical hallmark of apoptosis. Internucleosomal degradation of the genome // Cancer Metastasis Rev.— 1992.— **11**, N 2.— P. 105—119.
7. *Walker P. R., Kokileva L., LeBlanc J., Sikorska M.* Detection of the initial stages of DNA fragmentation in apoptosis // BioTechniques.— 1993.— **15**, N 6.— P. 1032—1040.
8. *Brown D. G., Sun X.-M., Cohen G. M.* Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation // J. Biol. Chem.— 1993.— **268**.— P. 3037—3039.
9. *Oberhammer F., Wilson J. W., Dive C. et al.* Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kB fragments prior to or in the absence of internucleosomal tragmentation // EMBO J.— 1993.— **12**.— P. 3679—3684.
10. *Cohen G. M., Sun X.-M., Fearnhead et al.* Formation of large molecular weight fragments of DNA is a key commitment step of apoptosis in thymocytes // J. Immunol.— 1994.— **153**.— P. 507—516.
11. *Heller C., Pohl F. M.* A systematic study of field inversion gel electrophoresis // Nucl. Acids Res.— 1989.— **17**.— P. 5989—6003.
12. *Solovyan V. T., Andreyev I. O., Kunakh V. A.* The fractionation of eukaryotic DNA by pulsed field gel electrophoresis. II. The discrete DNA fragments and the levels of chromatin structural organization // Mol. Biol. (Russian).— 1991.— **25**.— P. 1159.
13. *Nelson E. M., Tewey K. M., Liu L. F.* Mechanism of antitumor drug action: Poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-acridinylamino)-methansulphon-*m*-anisidide // Proc. Nat. Acad. Sci. USA.— 1984.— **81**.— P. 1361—1365.
14. *Osheroff N.* Biochemical basis for the interaction of type I and II topoisomerases with DNA // Pharmacol. Ther.— 1989.— **41**.— P. 223—241.
15. *Liu L. F.* DNA topoisomerase poisons as antitumor drugs // Annu. Rev. Biochem.— 1989.— **58**.— P. 351—375.
16. *Kaufman S. H.* Induction of endonucleolytic DNA cleavage in human acute myelogeneous leukemia cells by etoposide, camptothecin, and other anticancer drugs: A cautionary note // Cancer Res.— 1989.— **49**, N 21.— P. 5870—5878.
17. *Ucker D. S., Obermiller P. S., Eckhart W. et al.* Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic lymphocytes // Mol. Cell. Biol.— 1992.— **12**, N 7.— P. 3060—3069.
18. *Filipski J., Leblanc J., Youdale T. et al.* Periodicity of DNA folding in higfer order chromatin structure // EMBO J.— 1990.— **9**.— P. 1319—1327.
19. *Razin S. V., Petrov P., Hancock R.* Precise localization of the α-globin gene cluster within one of the 20- to 300-kilobase DNA fragments released by cleavage of chicken chromosomal DNA at topoisomerase II sites *in vivo:* Evidence that the fragments are DNA loops or domains // Proc. Nat. Acad. Sci. USA.— 1991.— **88**.— P. 8515—8519.
20. *Solovyan V. T., Andreyev I. O., Kunakh V. A.* The functional organization of plant nuclear DNA. I. Evidence for nuclear topoisomerase II/DNA complex // Mol. Biol. (Russian).— 1993.— **27**, N 6.— P. 1245—1251.

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