

COMPARATIVE CHARACTERISTICS OF DNA LOOP DOMAINS REARRANGEMENT IN GLIOBLASTOMA MULTIFORME T98G AND GLIOBLASTOMA ASTROCYTOMA U373 CELL LINES UNDER DIFFERENT CULTURE CONDITIONS

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Background: The loop domain organization of chromatin, which plays an important role in transcription regulation, may depend on the cell functional state. *The aim* of this work was to investigate DNA loop reorganization upon functional transitions in two cell lines – glioblastoma multiforme T98G and glioblastoma astrocytoma U373. **Materials and Methods:** Single cell gel electrophoresis (the comet assay) was used to analyze the kinetics of the DNA loop migration from the nucleoids obtained from the lysed cells. **Results:** The DNA fraction in the surface loops and the size of these loops were found to be similar in two glioblastoma cell lines. When synthetic processes were inhibited, the migration of a small portion of inner loops was observed in T98G but not U373 cells. In T98G cells, stimulation of cell proliferation and transcription was accompanied by an increase in DNA fraction in the inner loops and an essential increase in the size of these loops. The effect of stimulation was practically absent in U373 cells. However, the linear density of the loops resolved by the comet assay was found to decrease upon stimulation of proliferation in both cell lines. **Conclusion:** A decrease in the loop density appears to be associated with an intensification of the synthetic processes in cells upon their stimulation.

Key Words: DNA loops, comet assay, cell functional state, glioblastoma, T98G cell line, U373 cell line.

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Cell differentiation and de-differentiation including malignant transformation are accompanied by changes in the expression of particular genes as well as in the whole transcriptional profile of cells [1, 2]. It is known that the transcription regulation system relies on DNA loop domains — chromatin structures formed in the ATP-dependent process of loop extrusion [3–5]. As a result, gene promoters and their distal regulatory elements (enhancers or silencers) are brought into close physical proximity. Changes in the cell transcription level are associated with the reorganization of loop domains in the cell nuclei [5–7]. For example, using the Hi-C technique, the powerful approach for detecting genome-wide contacts between different regions of the chromatin fibers, it was shown that the activation of transcriptional processes in mouse B-lymphocytes is accompanied by an increase in the number of loops and their contour length [8].

In our previous works, we also focused on studying DNA loop dynamics in human peripheral blood lymphocytes upon their activation with recombinant interleukin 2 as well as in malignant lymphoid Jurkat cells [9, 10]. To assess the DNA loop domain organization, we applied the kinetic approach previously developed in our laboratory, which is based on the single-cell gel electrophoresis (the comet assay) of the isolated nucleoids [11, 12]. These structures are obtained after mild cell lysis in the presence of detergents and high salt. As our results showed, DNA loops in the nucleoids are in general the same as the chromatin loop domains [12], making the comet assay an easy and sensitive method for investigating DNA loop domain organization.

Analysis of the electrophoretic track (comet tail) formation allows us to evaluate a number of parameters of the loop organization in the cell nuclei. In particular, it enables to determine the amount of DNA in the loops localized in different regions of the nucleus (inside or on the surface) as well as the contour length and the linear density of these loops [12–14]. It was shown that the loop organization in activated lymphocytes and malignant lymphoid cells is similar. On the other hand, there are differences in comparison with terminally differentiated lymphocytes. First, DNA fraction in the loops of large sizes (more than ~200 kb) was essentially increased in the activated and transformed cells. Second, the linear density of the loops not larger than ~200 kb was decreased in these two types of cells under conditions that promote a high level of transcriptional activity while it increased upon the inhibition of synthetic processes [10].

In this work, we focused on the comparison of the DNA loop domain organization in two cell lines – glioblastoma multiforme T98G and glioblastoma astrocytoma U373 – in the setting of serum depletion followed by re-stimulation by serum addition. Both cell lines are frequently used as models for studying the mechanisms of cell division control [15, 16], the effects of test drugs [17], the expression of a number of tumor suppressor genes, etc. Despite the fact that the origin of these cells is similar, a number of characteristics significantly distinguish one line from another: ploidy, morphology, tumorigenicity, response to changes of culture conditions, etc. [15, 18–20].

The questions we have addressed here are (i) how different or similar the loop organization of these two cell lines is, and (ii) how the modulation of their transcription level is associated with the loop organization.

The obtained results indicate that in serum-depleted cells of both lines the amount of DNA in the surface loops and the contour length of these loops are similar. The migration of inner loops was observed only in the T98G cell line. Renewal of the cells proliferation and active synthesis (re-stimulation) was accompanied by a decrease in the loop density in both cell lines. In the T98G cells, in addition, an increase in the contour length of the inner loops and the amount of DNA in these loops was observed while any changes in these parameters were not detected in the U373 cell line.

MATERIALS AND METHODS

Cell lines. We used two types of tumor cell lines — human glioblastoma multiforme T98G and human glioblastoma astrocytoma U373, both cell lines are of glial origin. T98G is considered to be a polyploid derivative of the T98 cell line originated from human glioblastoma multiforme and possesses spherical, polygonal, and fibroblast-like shape [18]. Regarding cellular proliferation control, T98G combines features of both normal and malignant cells. Like normal cells, T98G is arrested in the G1 phase in a low serum-containing medium or at high cell density. At the same time, T98G cells are immortalized and anchorage-independent and are not tumorigenic in nude mice [15]. U373 is a human glioblastoma astrocytoma cell line. U373 was derived from astrocytoma grade IV/glioblastoma multiforme and established as a permanent cell line in 1973 [19, 20]. It is pleomorphic/astrocytoid in morphology and has a diploid karyotype [21]. It was shown that U373 cells are tumorigenic in athymic nude mice [20].

Cell culture. Cells were cultured at 37 °C in DMEM with 10% fetal bovine serum and antibiotics. In order to decrease cells transcription activity, partly synchronize the culture and, for T98G cells only, stimulate its arrest in the G1 phase, cells were put in the same medium without serum and incubated for 24 h or 48 h. For re-stimulation to proliferation, the cells were sedimented by centrifugation, put in DMEM medium with 10% fetal bovine serum, and cultured for 8 h or 16 h. The cells were collected by centrifugation, washed twice with Hanks' salt solution and used for the preparation of nucleoids.

The comet assay was performed as described earlier [11–13]. Briefly, cell suspension in Hanks' salt solution was mixed with 1% low-melting point agarose and an aliquote of the mixture was used to prepare a microscope slide covered with 1% high-melting point agarose. Slides were treated with ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1% Triton X-100) overnight, washed by TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 7.5) and subjected to electrophoresis in the same buffer. Several slides, which were simultaneously prepared in the same way, were placed into the electrophoresis tank, and then they were taken out every 5 or 10 min of electrophoresis. After electrophoresis, slides were stained with DAPI and immediately analyzed by a fluo-

rescent microscopy. A total of 100–150 randomly chosen nucleoids on each slide were examined using image analysis software CometScore (TriTek, USA) to determine the relative amount of DNA in the tails and the tail length. The tail length values were multiplied by two and divided by 0.34 nm to estimate the contour length of the longest loops in the tails.

Analysis of the kinetic plots. For T98G cells, the kinetic plots (the relative amount of DNA in the tail f vs. electrophoresis time t) were sigmoidal and they were fitted with the equation $f = A_1 f_1 + A_2 f_2$, where A_1 and A_2 are the maximum amplitudes of two components (see [12] for details). The function f_1 is described by a standard equation of monomolecular kinetics:

$$f_1 = 1 - \exp(-k_1 t), \quad (1)$$

where k_1 is the rate constant. The function f_2 obeys the sigmoidal Boltzmann equation:

$$f_2 = 1/[1 + \exp(k_2(t_0 - t))], \quad (2)$$

where k_2 is the rate constant, t_0 is the transition half-time. For U373 cells, it was possible to describe the plots by a monomolecular reaction scheme.

The dependences of the relative amount of DNA in the tail f on the contour length l_m of the longest loops in the tail were fitted with the equation [13]:

$$f = f_m [1 - (1 + \gamma l_m) \exp(-\gamma l_m)], \quad (3)$$

where f_m is the maximum relative amount of DNA that can exit and γ is the loop density (the number of loops per 1 kb).

RESULTS

Typical images of nucleoids prepared from T98G and U373 cells at different stages of electrophoresis are presented in Fig. 1. The appearance of the comets is about

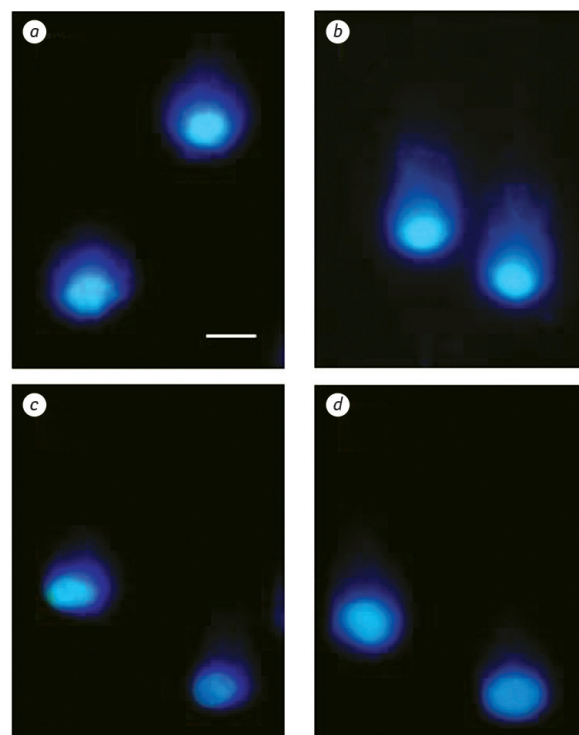


Fig. 1. Typical images of comets after 20 (a, c) and 60 (b, d) min of electrophoresis for T98G (a, b) and U373 (c, d) cell lines. Bar: 10 μ m

the same for cells of both lines and does not differ from other cell types that we investigated previously [11–13].

As we have shown earlier [22], for serum-depleted culture of T98G cells the kinetic plot of the average relative amount of DNA in the comet tails has a two-step shape (Fig. 2, a). At the first rapid step, which reflects the migration of DNA loops from the nucleoid surface [12], the amount of DNA in the tail does not exceed 10% of the total DNA amount in the nucleoid. Inner loops, whose exit into the comet tails occurs at the second step [12], give an additional increase (~5%) in the DNA fraction in the comet tails (Table).

In contrast to T98G cells, for U373 cells the kinetics of DNA exit into the comet tails occurs in one step and obeys monomolecular reaction scheme (Fig. 2, a, Table): the relative amount of DNA in the tails remains unchanged at the level of ~0.07 even after 80 min of electrophoresis. This value is comparable with the DNA amount achieved at the first step for T98G cells. Thus, the migration of inner DNA loops was not observed for U373 cells at all.

Along with the analysis of the DNA fraction in the inner and surface loops, the estimation of the contour length of the longest loops of the two types was performed (Fig. 3, a). The length of the surface loops in the comet tails is almost similar for both cell types and varies from ~20 to ~80 kb at different times of electrophoresis duration. As it was mentioned above, the exit of the inner loops was observed for

T98G cells only (the second step of the kinetic plot in Fig. 2, a) and the length of these loops increased with time up to ~150 kb.

It should be noticed that for both cell types, the comet tails contain only a small part of DNA in the nucleus (~13% and ~7% of the total genome for T98G and U373 cells, respectively). This observation points out that the loops, which cannot exit during electrophoresis, are probably the loops, which are too large.

The correlations between the length of the longest loops and the relative amount of DNA in the tails (Figs. 2, a, 3, a) allow one to estimate the loop density (γ in Eq. 3), the main parameter of the exponential distribution of the loop length [13]. Fitting Eq. 3 to dependences of f upon l_m gives close values of the density: $\gamma = 0.059 \pm 0.007 \text{ kb}^{-1}$ for T98G cells and $\gamma = 0.067 \pm 0.005 \text{ kb}^{-1}$ for U373 cells. Note that the loop density estimated is related only to the loops, the sizes of which are within the resolution of the comet assay.

To stimulate again the cell proliferation and transcription activity, serum was added to the medium and the kinetics of DNA exit from nucleoids was again examined. For re-stimulated T98G cells, a significant increase in DNA amount in the tails and in the length of the longest loops in the tails was observed at both steps of migration (Figs. 2, b, 3, b; Table). The increase was especially pronounced for the length of the inner loops: from ~150 to ~300 kb (Fig. 3). In contrast to T98G cells, both investigated parameters remained

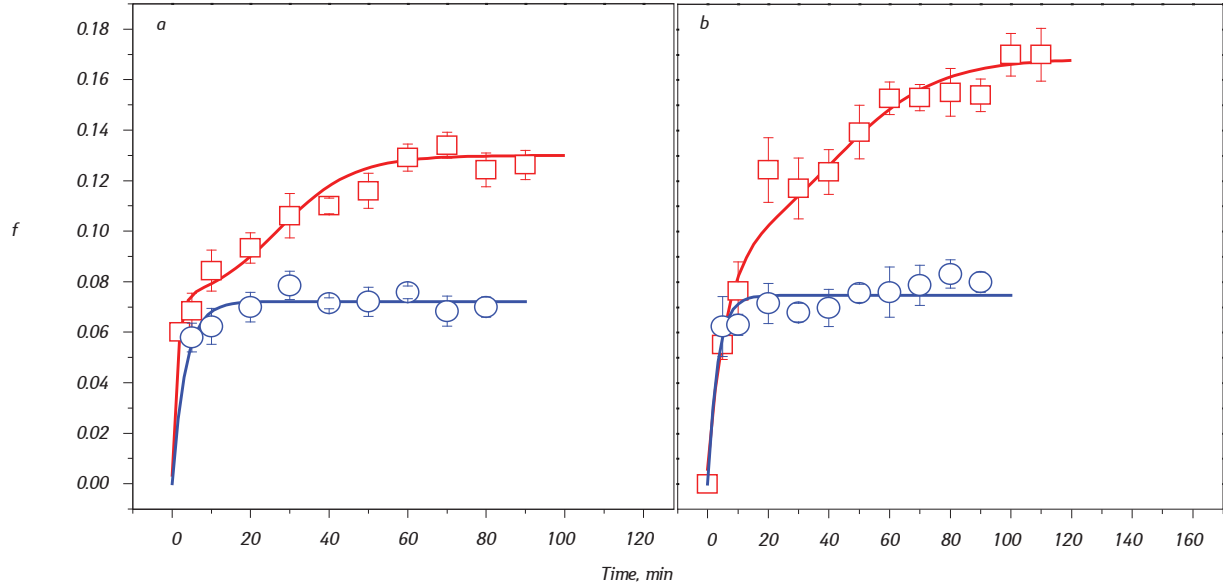


Fig. 2. The average relative amount f of DNA in the comet tails as functions of electrophoresis time for nucleoids obtained from serum-depleted (a) and re-stimulated to proliferation (b) T98G cells (□) and U373 cells (○). Here and in Fig. 3 below, each point is an average of 5 to 7 independent experiments, error bars are the standard errors

Table. Kinetic parameters of DNA exit from nucleoids derived from serum-depleted and re-stimulated to proliferation T98G and U373 cells

Kinetic parameter	T98G		U373	
	Serum-depleted	Re-stimulated	Serum-depleted	Re-stimulated
k_1, min^{-1}	0.66 ± 0.29	0.17 ± 0.05	0.29 ± 0.05	0.32 ± 0.07
k_2, min^{-1}	0.09 ± 0.04	0.06 ± 0.01	—	—
t_0, min	27 ± 8	41 ± 10	—	—
A_1	0.07 ± 0.01	0.09 ± 0.01	0.07 ± 0.001	0.07 ± 0.002
A_2	0.05 ± 0.01	0.08 ± 0.02	—	—

Notes: A_1 and A_2 are the maximum amplitudes of two components of the kinetic plots; k_1 and k_2 are the rate constants of the first and second step of the kinetic plot, respectively; t_0 is the transition half-time for the second step.

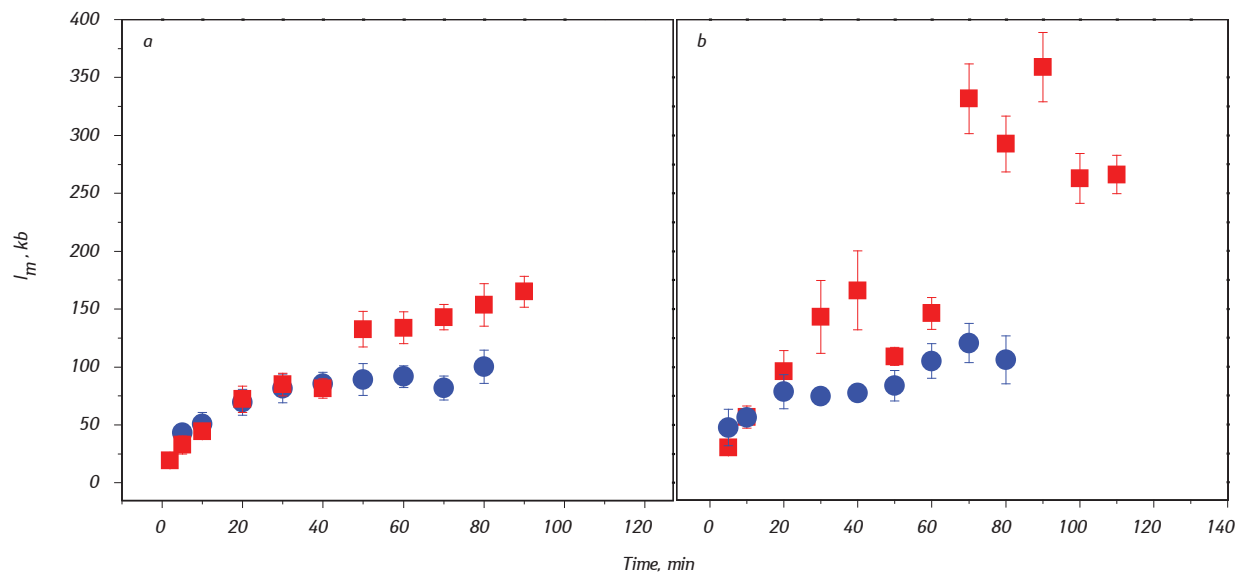


Fig. 3. The contour lengths of the longest loops in the tails (l_m) as functions of electrophoresis time for nucleoids obtained from serum-depleted (a) and re-stimulated to proliferation (b) T98G cells (■) and U373 cells (●)

almost the same for serum-depleted and re-stimulated U373 cell culture, although a little enlargement of the loops was observed at later times of electrophoresis (Fig. 3, b). For both cell lines, significant decrease in the loop density was observed after stimulation of proliferation. This value became almost two times lower for T98G cells ($\gamma = 0.025 \pm 0.002 \text{ kb}^{-1}$) and decreased to $0.053 \pm 0.003 \text{ kb}^{-1}$ for re-stimulated U373 cells.

DISCUSSION

Investigation of the kinetics of the comet tail formation during the comet assay makes it possible to estimate a number of parameters of the loop organization in nuclei [12, 14]. Earlier, we studied the physical mechanisms of the tail formation and demonstrated that the first rapid stage of DNA migration is provided by pulling the loops located on the nucleoid surface. These loops are characterized by the absence of supercoiling and a relatively small contour length [12].

In present study, we analyzed DNA loop reorganization in T98G and U373 glioblastoma cell lines in the setting of serum deprivation/restoration. For both serum-free cell cultures, the fraction of DNA in the surface loops is practically identical and coincides within the error with this parameter obtained, for example, for intact lymphocytes [9, 10].

It can be argued that the relative number of outer loops is a common characteristic of the cell nucleus of these two cell lines. The sizes of the outer loops are also similar and do not exceed ~ 80 kilobases. Based on general ideas about the structure of the nucleus, it can be assumed with some caution that the surface loops originate from constitutive heterochromatin (centromeric and telomeric regions which are known to be localized on the nuclear periphery), which explains the similarity of the DNA fraction in these loops.

As shown earlier, the DNA fraction in the inner loops of nucleoids, which can be visible in the comet assay,

is a more variable parameter [13]. Their migration is delayed and cooperative (as indicated by the S-shaped kinetics of their exit). These loops are supercoiled and rather large. In our experiments, migration of the inner loops was observed only for T98G cells, and the DNA fraction in their composition did not exceed $\sim 5\%$. For comparison, in human lymphocytes, the DNA fraction in such loops is about 14%, while in malignant lymphoid Jurkat cells as well as in U373 cells, the exit of these loops was not observed at all [10]. The rather small relative amount of DNA in the inner loops in T98G cells and the complete absence of their migration in U373 cells can be explained by the significant size of these loops.

The analysis of the correlation between relative DNA amount in the tails and the tail length makes it possible to determine the linear density of the loops, the sizes of which are within the resolution of the comet assay. For serum-depleted cells of both types, this value was similar within the error and comparable to that obtained, for example, for Jurkat cell line [10].

Re-adding serum to the culture medium activates suspended synthetic processes. This is accompanied by a change in the loop organization only in T98G cells and is especially pronounced for inner loops. This reorganization consists of an almost 1.5-fold increase in the fraction of DNA in the inner loops and a 2-fold increase in their size. It is curious that similar changes (an increase in the size of internal loops and the relative DNA amount in their composition) were also observed upon activation of lymphocytes by interleukin-2 [9]. The absence of visible changes in the amount of DNA in the comet tails and the contour length of the loops in U373 cells shows apparently that the inner loops in these cells are too large under any conditions.

It should be noted that T98G cells are arrested at the G1 phase of the cell cycle in serum-free medium [23]. In contrast, U373 cells retain some low level of synthetic activity in this setting. Our attempt

to induce the arrest by extending the incubation time in the serum-free medium above 48 h was not successful: the cells died. Perhaps this difference (which may be related to the different grade of malignancy) explains why any changes in the loop organization were not observed in U373 cells, while T98G cells exhibit changes in the loop organization, which are typical for normal cells (in particular, for lymphocytes) when the synthetic processes are stimulated one way or another.

Nevertheless, for both T98G and U373 cells, upon re-stimulation of proliferation, a decrease in the linear loop density is observed, which is much more pronounced for T98G cells. A similar effect was also observed upon activation of lymphocytes by interleukin-2.

An important question that has not been addressed is whether there are any cancer-associated changes in the loop domain organization. This issue remains largely unexplored and should be clarified by future research.

To sum up, the results of our comparative study of the two glioblastoma cell lines show the similarity in both the DNA fraction in the surface loops and in their contour length. When synthetic processes are inhibited, the migration of a small portion of inner loops, the contour length of which is comparable to that obtained for other cells investigated earlier, was observed in T98G but not U373 cells. In T98G cells, stimulation of transcription and cell proliferation was accompanied by an increase in the DNA fraction in the inner loops and an essential increase in the size of these loops. Such an effect was practically absent in U373 cells. However, the linear density of the loops resolved by the comet assay was found to be decreased upon re-stimulation of both cell lines.

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**ПОРІВНЯЛЬНА ХАРАКТЕРИСТИКА
РЕОРГАНІЗАЦІЇ ПЕТЕЛЬНИХ ДОМЕНІВ ДНК
В КЛІТИННИХ ЛІНІЯХ МУЛЬТИФОРМНОЇ
ГЛІОБЛАСТОМИ T98G ТА ГЛІОБЛАСТОМИ
АСТРОЦИТОМИ U373 ЗА РІЗНИХ УМОВ
КУЛЬТИВУВАННЯ**

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Вступ: Петельна організація хроматину, яка відіграє важливу роль у регуляції транскрипції, може залежати від функціонального стану клітин. **Мета** цієї роботи полягала у дослідженні реорганізації петель ДНК під час зміни функціонального стану клітин двох ліній — мультиформної гліобластоми T98G та гліобластоми астроцитоми U373. **Матеріали та методи:** Електрофорез ДНК ізольованих клі-

тин (кометний електрофорез) було використано для аналізу кінетики міграції петельних доменів ДНК з нуклеотидів, отриманих шляхом лізису клітин. **Результати:** Частка ДНК, яка припадає на поверхневі петлі, та розміри цих петель були схожими для двох ліній клітин гліобластоми. За умови інгібування синтетичних процесів міграція невеликої фракції внутрішніх петель спостерігалася лише для клітин лінії T98G. Для цих же клітин стимулювання клітинної проліферації та транскрипції супроводжувалося збільшенням частки внутрішніх петель і суттєвим збільшенням їх розмірів. Подібні ефекти стимулювання були майже повністю відсутні у клітин лінії U373. Проте лінійна щільність петель ДНК, які детектуються за допомогою кометного електрофорезу, знижувалася при стимулюванні проліферації в обох типах клітин. **Висновок:** Зниження щільності петель ДНК асоційоване з підвищенням інтенсивності синтетичних процесів у клітинах при їх стимуляції до поділу.

Ключові слова: петлі ДНК, кометний електрофорез, функціональний стан клітин, гліобластома, клітинна лінія T98G, клітинна лінія U373.