Journal of Cellular Biochemistry 104:692–699 (2008)

Repositioning of ETO Gene in Cells Treated With VP-16, an Inhibitor of DNA-Topoisomerase II

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Abstract The translocation t(8;21)(q22;q22) affecting AML1 and ETO genes is known to be one of the frequent chromosome translocations in acute myeloid leukemia. But no data have been available up to date concerning mutual positioning of these particular genes in the nucleus of a living cell as well as the mechanism of their rapprochement and realignment. Here we show that there is no proximity between these two genes in the primary nuclei of normal human male fibroblasts and moreover that these genes are located in different nuclear layers. But we further show that treatment of cells with VP-16 (etoposide), an inhibitor of DNA topoisomerase II widely used in anticancer chemotherapy, causes the ETO gene repositioning which allows AML1 and ETO genes to be localized in the same nuclear layer. Inhibitor studies demonstrate that such an effect is likely to be connected with the formation of stalled cleavable complexes on DNA. Finally, inhibition of ETO gene repositioning by 2,3-butanedione monoxime (BDM) suggests that this process depends on nuclear myosin. Together, our data corroborate the so called ''breakage first'' model of the origins of recurrent reciprocal translocation. J. Cell. Biochem. 104: 692–699, 2008.

Key words: topoisomerase II; translocations; nuclear repositioning; ETO gene

Recent investigations have shown that in many cases antitumor chemotherapy with topoisomerase II-specific agents results in secondary leukemias. These leukemias are often related with various chromosomal rearrangements which are usually considered to be the cause of neoplastic cell transformation [Rowley, 1993; Rowley et al., 1997; Felix, 1998; Andersen et al., 2001; Zhang et al., 2002; Zhang et al., 2004]. Detailed analysis of chromosomal rearrangements associated with secondary leukemias has permitted a number of genes participating in tumorogenic chromosomal translocations to be identified (reviewed by Bystritskiy and Razin [2004]). As demonstrated by analysis of translocation junctions, in each of translocation partners the breakpoints were clustered within relatively narrow areas named breakpoint cluster regions (BCRs) [Felix, 1998; Zhang et al., 2002; Zhang and Rowley, 2006]. The current model suggests that repair of double-stranded DNA breaks (DSB) induced by DNA toipoisomerase II proceeds for the most part through the low-fidelity non-homologous end-joining mechanism. Incorrect joining of cleaved DNA chains is likely to be the main cause of translocations occurring in cells treated with topoisomerase II poisons [Kantidze et al., 2006]. From a general point of view it seems clear that broken DNA chains of different chromosomes can be incorrectly joined only in the case of localization close to each other. In this connection, it is important to remind that in eukaryotic cell nuclei different chromosomes occupy specific non-overlapping spaces known as chromosome territories [Cremer and Cremer, 2001]. Furthermore, it was demonstrated that different chromosomes occupy specific nuclear layers, gene-rich chromosomes being located close to the nuclear center and gene-poor

Grant sponsor: Presidium of the Russian Academy of Sciences; Grant number: MCB; Grant sponsor: RFBR; Grant numbers: 06-04-48358a, 07-04-91556a.

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Received 11 September 2007; Accepted 7 November 2007
DOI 10.1002/jcb.21656

chromosomes—close to the nuclear periphery [Croft et al., 1999; Boyle et al., 2001; Cremer and Cremer, 2001]. Although in some cases the translocation partners are located close to each other in the nuclear space [Parada et al., 2002], this is not a general rule. One would expect translocations between chromosomes located in different nuclear layers to be very infrequent, and yet in some cases the well-known translocation partners are present in chromosomes that occupy distinct nuclear layers. Thus, the translocation t(8;21)(q22;q22) is one of the frequent chromosome translocations in acute myeloid leukemia [Zhang et al., 2002], although chromosome 21 bearing the AML1 gene is located relatively close to the nuclear center, while chromosome 8 bearing the ETO gene occupies a more peripheral nuclear position [Boyle et al., 2001]. Thus there should be a mechanism which brings these two chromosomes or at least the domains of these chromosomes which participate in the translocation event to the same nuclear region. In this study, we have analyzed nuclear localization of AML1 and ETO genes in primary human fibroblasts before and after treatment with VP-16, a known topoisomerase II poison. We have found that these two genes are located in different nuclear layers in non-treated cells. After treatment with VP-16, in a considerable fraction of cells bearing the replicated ETO gene the latter is relocated to the nuclear layer occupied by the AML1 gene. These results strongly support the hypothesis postulating that broken chromosomal regions separated by a long distance can be subsequently brought together to produce translocations [Aten et al., 2004].

MATERIALS AND METHODS

Cell Culture and Preparation of Microscopic Slides With Fixed Cells

The culture of primary embryonic normal human male fibroblasts was received from the Institute of Medical Genetics of the Russian Academy of Medical Sciences. The cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum. Fluorescence in situ hybridization (FISH) analyses were performed after 3–4 passages. Normal human male fibroblasts (HEF1698) were incubated for 1.5 h in the presence of 0.17 mM of VP-16 or 0.17 mM of merbarone or 0.17 mM of camptothecine or 0.17 mM of VP-16 together with 20 mM of 2, 3-butanedione monoxime (BDM). After incubation the medium was replaced by a pure medium, and the cells were incubated for 3 more hours in order to attain a vague recovery. Before fixation, the cells grown on microscopic slides were permeabilized on ice in a buffer containing 10 mM Pipes (pH 7.8), 100 mM NaCl, 3 mM $MgCl_2$, 0.5 mM $CuSO_4$, 0.2 mM PMSF (phenylmethylsulfonyl fluoride), 300 mM sucrose, 0.5% (w/v) Triton X-100 for 10 min. The cell samples were then immediately fixed in 4% paraformaldehyde in 1 × PBS (pH 7.4) for 15 min at room temperature, dehydrated in cold 70, 80, and 96% ethanol and air-dried and then stored at 4°C until use.

Visualization of AML1 and ETO Genes by Using Fluorescence In Situ Hybridization

The probes for AML1 and ETO genes were obtained from BAC clones RP1-140K16 and RP11-777J24 (CHORI) respectively. FISH was carried out as described [Iarovaia et al., 2004a,b] with slight modifications aimed to adapt the procedure to cells growing in monolayer. The cell samples on microscopic slides were treated sequentially with RNase A (100 μg/ml in 2 × SSC) and with pepsin (0.001% in 10 mM HCl), post-fixed with 1% paraformaldehyde and rinsed sequentially in 70, 80, and 96% ethanol.

The hybridization probes were labeled either with biotin or with digoxigenin-11-dUTP using a random-prime labeling kit (Roche, Switzerland). The hybridization mixture contained (in a final volume of 10 μl 65% (v/v) formamide, 2 × SSC, 10% dextran-sulfate, 0.1% Tween-20, 10 μg of Cot1 human DNA (Sigma), 2 μg of yeast tRNA and 25–50 ng of the labeled probe. Before hybridization, the mixture together with the cell preparation was incubated for 5 min at 74°C under the coverslip to denature DNA. Hybridization was carried out overnight at 37°C. After hybridization, the samples were washed for 30 min each in 50% formamide-2 × SSC at 40°C, in 2 × SSC at room temperature, and finally in l × SSC at room temperature. The labeled probes were visualized using either anti-digoxigenin monoclonal antibodies conjugated with FITC (Sigma) with subsequent signal amplification using an Alexa 488 signal amplification kit for mouse antibodies (Molecular Probes) or with neutravidin–rhodamin with sequential amplification of the signal using biotinilated goat antiavidin. In all

cases the DNA was counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). The results were examined under a fluorescence microscope DMR/HC5 (Leica) equipped with an objective HCX PZ Fluotar 100^{X}/1.3 and recorded using a CCD camera DC 350 F (Leica).

Computer-Assisted Analysis of Microscopic Images

The microscopic images were analyzed using the computer software "Nucleus Marker v3.1" described previously [Petrova et al., 2005]. This software permitted sequential treatment of three photographs of one and the same field taken with filters allowing DAPI-stained nuclei, rhodamine-stained, and Alexa 488-stained hybridization signals to be distinguished. The nuclear borders are defined and outlined by analyzing the intensity of DAPI staining. To analyze the distribution of the signals among the nuclear layers, vectors from the nuclear mass center to the hybridization signals are constructed for each nucleus. The vectors for each hybridization signal are extended to the nuclear border and the percent ratio of the first vector to the second one is calculated. The results are presented in the tabulated form in the Microsoft Excel format. To evaluate the distances between hybridization signals corresponding to AML1 and ETO genes, vectors from the hybridization signals of one type to the hybridization signals of the second type are constructed for each nucleus and the total area is calculated for each nucleus. The length of the resulting vectors is normalized to the radius of a theoretical circle having the same area as the area found for each examined nucleus.

RESULTS

Analysis of Mutual Localization of AML1 and ETO Genes in Primary Human Fibroblasts

We have first analyzed the mutual localization of AML1 and ETO genes in primary human fibroblasts. The distribution of signals was analyzed using flattened samples as this kind of analysis was reported previously to give to a first approximation reliable information about nuclear positions of chromosome territories, especially in relatively flat and long nuclei of primary fibroblasts. [Boyle et al., 2001; Zink et al., 2004].

The computer software used to determine the positions of hybridization signals within nuclei (Nucleus Marker v3.1) permits one to establish distances between an identified signal of each type and thus to calculate distances between AML and ETO genes in the nucleus. The obtained results (Fig. 1) demonstrate that the distribution of distances between AML1 and ETO signals does differ from the random one (distribution of distances between two random points in the circle for 1,000 events). Thus at least in the greater part of cells present in the population of primary human fibroblasts the AML1 and ETO genes are located much closer to each other as compared with such a theoretical situation when these genes are uniformly distributed within the nuclei and there exist an equal probability to meet each of these genes in any given spot of intranuclear space. But observed relative proximity of these genes to each other, in our opinion, does not necessarily reflect their actual proximity in terms of their localization in the same nuclear layer. It might be due to certain restrictions imposed on the nuclear localization of the genes by chromosomal territories which are known to have preferential positions in the nuclear space (see

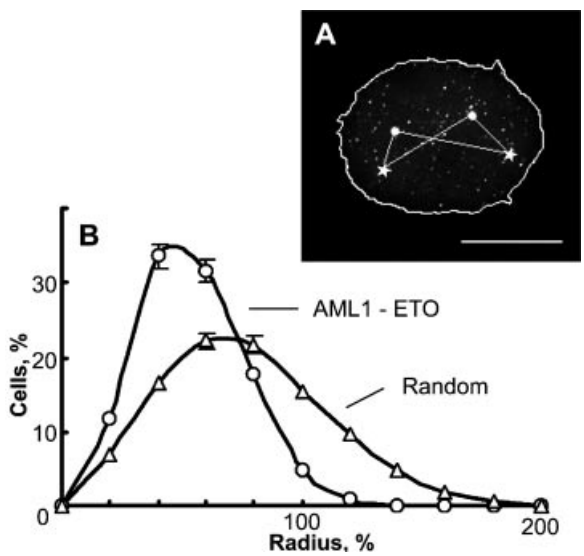

Fig. 1. **A**: The principle of measuring the distances between AML1 (white dots) and ETO (white stars) hybridization signals. Bar, 10 μm. **B**: Distribution of distances between AML1 and ETO signals (open circles) and between two random spots (open triangles). The values on abscissa show normalized distances between the AML1 and ETO genes. Values on ordinate represent the percentage of cells where the determined distances between the signals fall within the interval indicated on abscissa. Bars, standard deviation. The graph is based on the results of analysis of mutual distribution of AML1 and ETO genes in 260 cells (1,040 events).

Discussion). That is why we next asked ourselves a question as to whether the AML1 and ETO genes are located in the same or in different nuclear layers. Therefore, the distribution of hybridization signals among five concentric shells of the same area (with the inner borders of the shells located at 0, 45, 63.2, 77.5, and 89.4 percent of the nuclear radius) was analyzed. The results presented in Figure 2 show that the AML1 gene is located closer to the nuclear center as compared to the ETO gene. Indeed 96% of the AML1 gene and only 52% of the ETO gene occurred in the central part of the nucleus comprising 50% of the nuclear area. At the same time, it is important to mention that the ETO gene was characterized by a wider radial distribution. Due to this fact a portion of this gene was present in the same layer with the AML1 gene. One may suppose that the wide distribution of the ETO gene among the nuclear layers reflects the fact that the asynchronous cell population analyzed in the above-described experiments is composed of sub-populations characterized by different radial positions of the ETO gene. If so, these sub-populations might be related to the stages of the cell cycle. FACS analysis demonstrated that 60% of cells present in our population resided in G0 or G1 phases (Fig. 3A). This can be expected for a monolayer population of normal cells which display contact inhibition. About 38% of cells were characterized by 2× (13%) or intermediate (25%) DNA content, that is, were in G2 or S phases of the cycle. In agreement with these results about 25% of cells incorporated BrdU (bromodeoxyuridine) and showed early (~10%), late (~12%), or intermediate (~3%) replication patterns (Fig. 3B). It is likely that cycling and resting cells would react quite differently to treatment with topoisomerase II poisons. In order to test this possibility we analyzed the distribution of the replicated ETO gene among five concentric nuclear shells of equal area. The replicated gene can easily be discriminated because of characteristic double signals [Handeli et al., 1989]. The results of the analysis

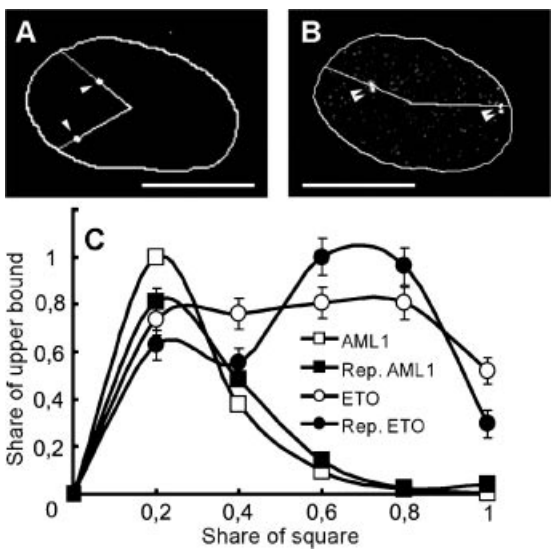

Fig. 2. **A**, **B**: Illustration of the principle of evaluation of the radial distribution of single (arrowheads) and double (double-arrowheads) hybridization signals within nuclei. Bar, 10 μm. **C**: The distribution of hybridization signals representing AML1 (open squares), replicated AML1 (closed squares), ETO (open circles), and replicated ETO (closed circles) among five concentric shells of the same area. Graphs demonstrating distribution of AML1 and ETO genes are based on the results of analysis of 250 cells (500 measured distances for each gene). Graphs demonstrating distribution of replicated AML1 and replicated ETO genes are based on the results of analysis of 60 and 50 cells respectively. Bars, standard deviation. Note that the AML1 gene is located closer to the nuclear center as compared to the ETO gene.

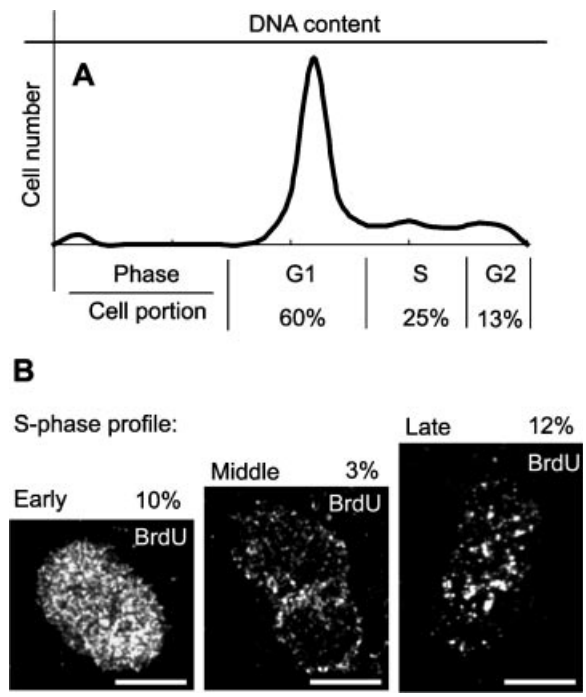

Fig. 3. **A**: Results of FACS analysis of normal human male fibroblasts. **B**: Different replication patterns observed in normal human male fibroblasts. The cells were pulsed with BrdU (50 μg/ml, 30 min) with the following chase for 1 h, fixed routinely with formaldehyde, and stained with BrdU-specific antibodies conjugated with FITS (Images, bar 10 μm). The percentage of cells showing early, intermediate, and late replication patterns is indicated above the images.

show that the radial distribution of the replicated ETO gene is clearly bimodal (χ^2, $P < 0.05$), with the minor peak being located in the same nuclear layer as is the AML1 gene. As for the AML1 gene, P value in χ^2 test came to more than 0,2 and the distribution of replicated copies was practically the same as compared to the distribution of this gene in the whole cell population.

Inhibition of Ligation Activity of DNA Topoisomerase II Induces a Shift of ETO Gene Toward the Nuclear Center

Topoisomerase II acts as a regulator of DNA topology. It can relax positive and negative supercoils in circular DNA (or closed loop domains of eukaryotic DNA) and unknot or uncouple DNA molecules by introducing transient double-strand breaks [Wang, 2002]. Normally such breaks are religated after unknotting of DNA molecules. However, many topoisomerase II inhibitors such as doxorubicin, amsacrine, teniposide, or etoposide block the catalytic reaction at the stage of a cleavable complex and thus introduce potential DSB. These drugs are known as topoisomerase II poisons. Other topoisomerase II-specific inhibitors such as merbarone act in a different way and do not stimulate accumulation of stalled cleavable complexes [D'Arpa and Liu, 1989; Liu, 1989; Chen and Liu, 1994].

In order to find out whether treatment of cells with different inhibitors of DNA topoisomerase II stimulates nuclear relocation of the ETO gene, we repeated the experiments described in the previous section using cells treated for 1.5 h either with VP-16 that stimulates accumulation of arrested cleavable complexes or with merbarone that suppresses activity of the enzyme without arresting cleavable complexes. The most interesting results were obtained with VP-16-treated cells. Already when the whole cell population was analyzed a shift of the ETO gene toward the nuclear center was observed, and the radial distribution of this gene became clearly bimodal (χ^2, $P < 0.05$) (Fig. 4A). This shift became much more obvious when only cells bearing the replicated ETO gene were taken into consideration (χ^2, $P < 0.05$). In these cells the ETO gene was almost equally distributed between two nuclear layers, one containing the AML1 gene and one located significantly closer to the nuclear periphery. The radial position of the AML1 gene did not change after treatment

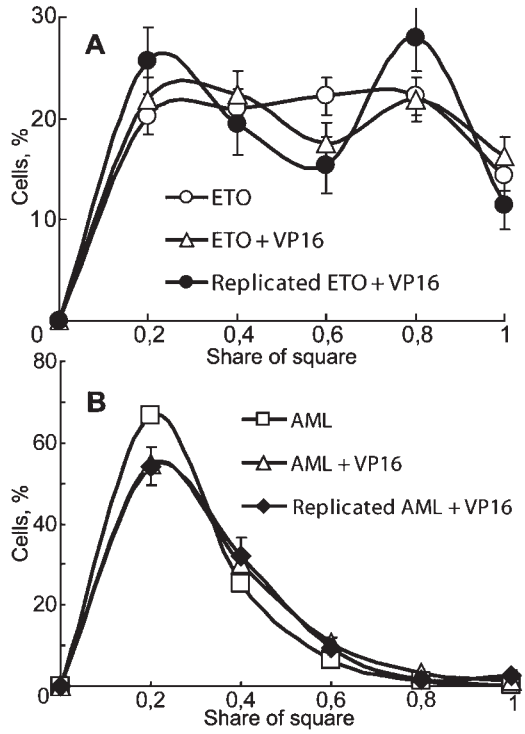

Fig. 4. Distribution of ETO and AML1 genes among five concentric nuclear shells of the same area in normal human male fibroblasts treated with VP-16. **A**: Distribution of hybridization signals representing the ETO gene in control (VP-16 untreated) cells (open circles) and in cells pre-treated with VP-16 (open triangles). Distribution of the replicated ETO gene in cells pre-treated with VP-16 is shown by closed circles. Nuclear positions of ETO gene after treatment of cells with VP-16 were determined in 180 cells including 87 cells with replicated ETO gene. **B**: Distribution of hybridization signals representing the AML1 gene in control (VP-16 untreated) cells (open squares) and in cells pre-treated with VP-16 (open triangles). Distribution of the replicated AML1 gene in cells pre-treated with VP-16 is shown by closed rhombuses. Bars, standard deviation. Nuclear positions of AML1 gene after treatment of cells with VP-16 were determined in 180 cells including 60 cells with replicated ETO gene.

of cells with VP-16 (Fig. 4B). Treatment of cells with merbarone induced a slight positional shift of the ETO gene toward the nuclear center but the distribution did not become clearly bimodal as in the case of cells treated with VP-16 (Fig. 5) (χ^2, $P < 0.05$). In contrast, treatment of cells with camptotecin, an inhibitor of DNA topoisomerase I that arrests cleavable complexes, had the effect comparable with that of VP-16 (Fig. 5) (χ^2, $P < 0.05$). It is thus likely that the presence of stalled cleavable complexes on DNA (independently of the nature of the enzyme) stimulates repositioning of the ETO gene.

This tentative conclusion was further supported by the analysis of mutual distribution of AML1 and ETO genes in cells treated

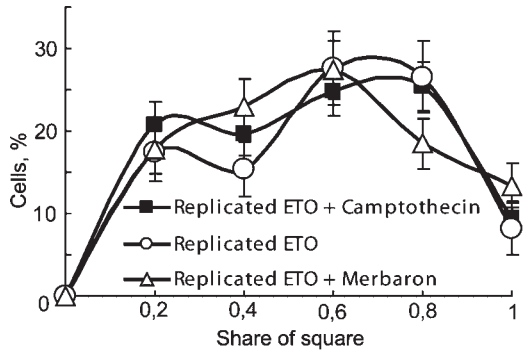

Fig. 5. Distribution of the replicated ETO gene among five concentric nuclear shells of the same area in normal human male fibroblasts treated with camptothecine (closed squares) or merbarone (open triangles). Distribution of the ETO gene among the same nuclear shells in control cells is shown by closed squares. Bars, standard deviation. Nuclear positions of ETO gene after treatment of cells with camptothecine or merbarone were determined in 110 and 80 cells respectively.

with VP-16. Experiments similar to these described in the first section of the Results demonstrated the direct increase in the portion of cells characterized by very short distances between AML1 and ETO genes in cells treated with VP-16. The portion of cells characterized by localization of AML1 and ETO genes at a distance of less than 5% of the nuclear radius increased by 1.7 times. The portion of cells characterized by distances between AML1 and ETO genes of less than 10% and less than 15% increased by 1.5 and 1.2 times respectively.

One may suppose that above-mentioned changes in nuclear localization of ETO gene are due to the beginning of apoptosis associated with an overall chromatin condensation. To exclude this possibility, we first analyzed the percentage of trypan blue-positive cells in normal population and after treatment of cells for 1.5 h with VP-16. No increase in the percentage of trypan blue-positive (dead) cells was observed at 3 h after VP-16 treatment (i.e., at the moment when the cells were fixed for the subsequent analysis in all our experiments). This conclusion was further confirmed by FACS analysis (data not shown). To check if the treatment of cells with VP-16 induces an overall chromatin condensation, the cells were immunostained with antibodies against lamin A (UCSC) and counterstained with DAPI. No signs of additional chromatin condensation in cells treated with VP-16 were observed (data not shown).

In the final set of experiments we tried to disclose the nature of mechanisms involved in the above-described repositioning of the ETO gene. To this end the cells were treated with VP-16 in the presence of BDM, a putative inhibitor of nuclear myosin [Aten et al., 2004]. The results (Fig. 6) demonstrated that repositioning of the ETO gene was significantly although not completely suppressed by BDM. This suggests the involvement of myosin motors in ETO gene repositioning.

DISCUSSION

Treatment of cells with inhibitors of DNA topoisomerase II frequently results in the induction of different chromosomal aberrations, some of them being associated with the development of leukemias. It is likely that chromosomal translocations are generated as a result of incorrect repair of DSB [Elliott and Jasin, 2002; Agarwal et al., 2006; Kantidze et al., 2006]. To be incorrectly joined the broken ends of different chromosomes should first meet in the nucleus. However, different chromosomes are not located at random within the nuclear space [Croft et al., 1999; Boyle et al., 2001; Cremer and Cremer, 2001]. Specific radial distribution of chromosome territories should put certain restrictions on the mobility of most genes and consequently on the probability of translocations between different chromosomes [Taslerova et al., 2003]. It was proposed that spatial positions of chromosomes and sub-chromosomal domains within

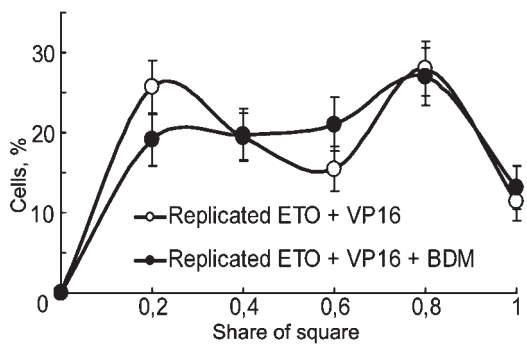

Fig. 6. Partial inhibition of ETO gene repositioning by BDM. Normal human male fibroblasts were incubated for 1.5 h in the presence of 0.17 mM of VP-16 or in the presence of 0.17 mM of VP-16 and 20 mM of BDM, whereupon the medium was replaced by a pure medium, and the cells were incubated for 3 more hours in order to attain a vague recovery. Distribution of the replicated ETO gene in cells treated with VP-16 (control experiment) and with VP-16 in the presence of BDM is shown by open and closed circles, respectively. Bars, standard deviation. Nuclear positions of ETO gene after treatment of cells with VP-16 in presence of BDM were determined in 80 cells.

nuclei determined preferential sites of translocations [Nikiforova et al., 2000; Savage, 2000]. Indeed, in some cases translocation partners are positioned in close spatial proximity relative to each other [Lukasova et al., 1997; Roix et al., 2003; Roccato et al., 2005]. However, this is not a general rule. In other cases no relation between spatial proximity of translocation partners and occurrence of the translocation was observed [Gue et al., 2006]. The so called "breakage first" model was proposed in order to account for the above discrepancy. According to this model the broken chromosome ends can migrate over large distances within nuclei. Such migration was indeed observed in nuclei subjected to irradiation with α-particles [Aten et al., 2004]. Furthermore, indirect evidence suggested that the movement of broken chromosome ends was mediated in an active fashion by actin–myosin motors [Aten et al., 2004]. However, in other studies contradictory results were obtained. It was found that broken chromosome ends were positionally stable within nuclei [Soutoglou et al., 2007].

AML1 and ETO genes studied in the present work are located in different nuclear layers. Nevertheless translocations between these genes are typical for secondary leukemias occurring after anticancer chemotherapy with topoisomerase II-specific drugs. We have found that distances between the AML1 and ETO genes in the nuclei of normal mail fibroblasts are shorter than one would expect basing on the assumption that both genes are randomly distributed. This may reflect the fact that localization of AML1 gene is restricted to the central part of a nucleus. Already for this reason the distances between AML1 and ETO gene cannot exceed significantly the length of the nuclear radius. Most interesting, we have demonstrated that after inhibition of topoisomerase II religation activity, that is, under conditions triggering activation of DSB repair by error-prone non-homologous end-joining [Kantidze et al., 2006], a portion of the ETO gene is repositioned to the more central nuclear layer where the AML1 gene is located. This result fits well the main premise of the above-discussed "breakage-first" model and provides a good explanation for the high recombination frequency between AML1 and ETO genes. It is of interest that repositioning of the ETO gene toward the nuclear center occurs predominantly in cycling cells. After treatment with VP-16, about 50% of the ETO gene was found in the same nuclear layer with the AML gene in cycling cells. It may be related to the fact that the consequences of DNA topoisomerase II inhibition are much more severe for cycling than for the resting cells. The mechanisms mediating apparent repositioning of the ETO gene remain obscure. To this end it is necessary to underline that the distribution of the ETO gene among the nuclear layers is fairly wide so that a portion of this gene is present in the same nuclear layer with the AML gene even in cells non-treated with topoisomerase II poisons. Spatial distribution of chromosome territories within nuclei is likely to be dynamic rather than static [Spector, 2003]. Simple fixation of stalled topoisomerase II cleavable complexes and/or stalled replication forks in the central part of nuclei would account for the observed shift toward the bimodal distribution of the ETO gene in replicating fibroblasts treated with topoisomerase II poisons. This fixation might occur because of non-random distribution within the nuclear volume of enzymes involved in processing of cleavable complexes and in non-homologous end-joining. Another possibility is that stalled topoisomerase II cleavable complexes are repositioned toward the central part of the nucleus in an active fashion. Inhibition of this repositioning by BDM, an inhibitor of nuclear myosin [Aten et al., 2004] corroborates this idea. Unfortunately, we were not able to check if repositioning of the ETO gene is blocked by actin depolymerization, as treatments of cells with cytochalasin B or latrunkulin D caused a drastic change in the cell shape. Thus further studies are necessary to disclose the mechanism of ETO gene repositioning occurring due to topoisomerase II inhibition. If indeed the damaged ETO gene is repositioned in an active fashion toward the nuclear center there should be some reason for that. One may speculate that proteins involved in processing of stalled topoisomerase II complexes are immobilized on the nuclear matrix in the central part of the nucleus and thus cannot reach the damaged genes located at peripheral nuclear layer.

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