Induction of transcription within chromosomal DNA loops flanked by MAR elements causes an association of loop DNA with the nuclear matrix

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ABSTRACT

The spatial organization of an ∼170 kb region of human chromosome 19, including CD22 and GPR40–GPR43 genes, was studied using in situ hybridization of a set of cosmid and PAC probes with nuclear halos prepared from proliferating and differentiated HL60 cells. The whole region under study was found to be looped out into the nuclear halo in proliferating cells. It is likely that the loop observed was attached to the nuclear matrix via MAR elements present at the flanks of the area under study. Upon dimethyl sulfoxide-induced differentiation of the cells the looped fragment became associated with the nuclear matrix. This change in the spatial organization correlated with the activation of transcription of at least two (CD22 and GPR43) genes present within the loop. The data obtained are discussed in the framework of the hypothesis postulating that the spatial organization of chromosomal DNA is maintained via constitutive (basic) and facultative (transcription-related) interactions of the latter with the nuclear matrix.

INTRODUCTION

In eukaryotic chromosomes DNA is organized into large loops fixed at the nuclear matrix. These loops were first observed in histone-depleted nuclei and metaphase chromosomes analyzed under an electron microscope (1–3). In histone-depleted nuclei observed under a fluorescence microscope DNA loops are seen as a crown surrounding the residual nuclear structure (nuclear matrix). This crown of loops is frequently referred to as a ‘nuclear halo’ (4–9). Individual DNA loops may be visualized using in situ hybridization of corresponding DNA probes with nuclear halos. In particular, in our previous study of the domain organization of the human dystrophin gene, a DNA loop mapped according to the topoisomerase II-mediated excision protocol was visualized using hybridization of an appropriate BAC probe with nuclear halos (10). In the present study we used the same approach in order to find out (i) whether matrix association regions [MARs (11)] are located at the basements of DNA fragments that are looped out in nuclear halos and (ii) whether the configuration of DNA loops changes upon induction of transcription of the genes present in these loops. The FXYD5-COX7A1 locus of human chromosome 19 was used as a model system. Distribution of MAR elements within this region was studied previously (12). Within the locus under study we selected an ∼170 kb region containing several genes flanked by MAR elements. Using in situ hybridization with nuclear halos we have demonstrated that this region is organized into a single DNA loop attached to the nuclear matrix apparently via MAR elements. In the case of one of the MAR elements flanking the area under study at the downstream end, this was directly confirmed using topoisomerase II-mediated DNA loop excision. Genes present within the loop are silent in proliferating HL60 cells but some of these genes are activated upon induced lymphoid differentiation of HL60 cells. We have found that under these conditions the whole area under study is not looped out in the nuclear halo, but remains associated with the nuclear matrix after high salt extraction. This finding supports the previously formulated hypothesis about the existence of constitutive (basic) and facultative (transcription-related) associations of DNA with the nuclear matrix (13,14).

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MATERIALS AND METHODS

Cell culture

Human acute promyelocytic leukemia HL60 cells (ATCC CCL-240) were grown in RPMI1640 with 10% fetal calf serum and stimulated for granulocyte-like differentiation by adding 1.5% dimethyl sulfoxide (DMSO) to the growth medium for 48 h.

DNA clones

Cosmid clones AC002132, U62631, AC002997 and a PAC clone AC002511 covering a region of human chromosome 19, which includes CD22 and GPR40–43 genes (15), were provided by A. Olsen, Lawrence Livermore National Laboratory, USA.

Preparation of nuclear halos

The cells were pelleted (700 g, 5 min), washed twice with RPMI medium and resuspended in permeabilization buffer [10 mM PIPES, pH 7.8, 100 mM NaCl, 3 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM CuSO4·300 mM sucrose and 0.5% (v/v) Triton X-100] at a final concentration of 2 × 106 cells/ml. After 4 min incubation on ice, the cells were cytosponned onto silane-coated microscope slides. The cells on the slides were then treated (4 min, 0°C) with high-salt solution [2 M NaCl, 10 mM PIPES, pH 6.8, 10 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.1% (w/v) digitonin] and after that sequentially washed (1 min each washing) with 10×, 5×, 2× and 1× PBS and then with 10, 30, 70 and 96% ethanol, air-dried, fixed in methanol–acetic acid (3:1) mixture, and dried at 70°C for 1 h.

In situ hybridization, immunolabeling and microscopy

The nuclear halos were treated sequentially with RNase A (100 µg/ml in 2X SSC) and pepsin (0.01% in 10 mM HCl), post-fixed with 1% paraformaldehyde and rinsed sequentially in 70, 80 and 96% ethanol. To denature DNA the slides were incubated in 70% formamide, 2X SSC for 5 min at 74°C, dehydrated in cold 70, 80 and 96% ethanol, and air-dried.

The probes were labeled with biotin-16-dUTP using a random-prime labeling kit (Roche). The hybridization mixture contained (in a final volume of 10 µl) 50% (v/v) formamide, 2X SSC, 10% dextran sulphate, 0.1% Tween-20, 10 µg of sonicated salmon sperm DNA, 10 µg of yeast tRNA, 25 µg of human Cot1 DNA (Sigma) and 25–50 ng of the labeled probe. Before hybridization, the mixture was incubated for 10 min at 74°C to denature DNA. The denaturation was followed by incubation at 37°C for 1 h. Hybridization was carried out overnight at 40–45°C. After hybridization, the samples were washed twice in 50% formamide and 2X SSC at 43–48°C for 20 min.

The biotinylated probes were visualized using anti-biotin monoclonal antibodies conjugated with Alexa 488 (Molecular Probes) with subsequent signal amplification using an Alexa 488 signal amplification kit for mouse antibodies and two additional layers of antibodies (chicken anti-goat and goat anti-chicken), both conjugated with Alexa 488. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The results were examined under a DMR/HC5 fluorescence microscope (Leica) equipped with a HCX PZ Fluotar 100X/1.3 objective and recorded using a CCD DC 350 F camera (Leica).

PCR-stop experiments

Proliferating HL60 cells were exposed to a topoisomerase drug, VM-26 (50 µg/ml), for 2 or 5 h. DNA samples isolated from control (non-treated) cells and from cells treated with VM-26 were used as templates to amplify a DNA fragment derived from the middle of the loop and a DNA fragment containing MAR 4. The amplification products were analyzed by agarose gel electrophoresis. The following pairs of primers were used for PCR amplifications: MAR4 fragment—direct primer 5'-GAGAACAAGCTGGCTGGGAGAGTAA-3', reverse primer 5'-TGATGGGATGTCAATTTGATG- loop fragment—direct primer 5'-CTCTCCCTCTTCTCCTC- TTCCCT-3', reverse primer 5'-ACCGACTACAGGACATCTACCT- CTC-3'.

RNA isolation, cDNA synthesis and RT–PCR

Total cellular RNA was isolated with the use of an RNeasy Mini RNA purification kit (Qiagen). All RNA samples were treated with DNase I to remove residual DNA. The first strand cDNA synthesis was performed using a SuperScript Preamplification System (Life Technologies-GibcoBRL). Control samples without reverse transcriptase were prepared in parallel. The efficiency of cDNA synthesis checked by means of RT–PCR using primers specific for the β-actin gene (Gene Checker Kit, Invitrogen) was equal in all preparations. RT–PCR was performed using a cDNA template obtained from 60 ng of initial RNA and two pairs of primers targeted at exons of the corresponding genes (5'-ATCGGCTCTCTGTGATGTA and 5'-GGGATGAAGAAGACAGGAC for GPR43; 5'-CTCTAGGAGACAGGAG for GPR40; 5'-CTCTAAGACAGCAGGACAG for GPR42; 5'-GATAACCGCAGTCAAAGG and 5'-GCTCTCCAGCAGGAG for MAG). Each reaction was carried out with 2.5 U of Platinum Taq DNA polymerase (Life Technologies-GibcoBRL) in 50 µl of the manufacturer’s buffer supplemented with 200 µM of each dNTP and 1.5 mM MgCl2 using the following profile: 94°C for 30 s, 60/65°C for 30 s, and 72°C for 45 s. The number of PCR cycles was in the range of 24–36 and depended on the level of transcription of the corresponding gene.

RESULTS

Visualization of a single DNA loop flanked by MAR elements

The map of the genomic region studied in this work is shown in Figure 1. It contains the CD22 gene composed of 15 exons (16) and a cluster of G protein-coupled receptor genes (GPR genes) of which three genes do not contain introns (17). The region is flanked by two MAR elements at the upstream end and by two MAR elements at the downstream end. In order to find out whether this region is organized into a single loop we carried out an in situ hybridization with nuclear halos of four DNA probes (clones AC002132, U62631, AC002511 and AC002997) covering the whole region under study and the flanking sequences (see the map in Figure 1). Nuclear halos representing the crown of extended loops fixed at the nuclear
matrix were prepared by 2 M NaCl extraction of HL60 cells cytospinned on microscopic slides (see Materials and Methods for details). The results of hybridization are shown in Figure 2. In most of the examined cells two loops extending from the nuclear matrix were clearly seen. Apparently, these loops represent genomic regions under study present in homologous chromosomes. Taking into consideration the area covered by the clones used as hybridization probes, one can conclude

**Figure 1.** A scheme of the area under study. Genes are shown by open rectangles above the scale line. MAR elements are shown by vertical bars. Numeration of MARs is according to ref. (12). Short horizontal bars show positions of two DNA fragments studied in PCR-stop experiments. Insertions of clones used for in situ hybridization experiments are shown below the scale line. The accession numbers of the corresponding DNA sequences deposited in the Gene Bank (AC002132, U62631, AC002511 and AC002997) are presented above the lines showing the insertions.

**Figure 2.** Visualization of the region under study on nuclear halos prepared from non-induced HL60 cells. Panels A–D show DAPI staining of nuclear halos and the results of hybridization are shown in panels A′–D′. Panels A″–D″ show superposition of DAPI staining and hybridization signals, which are seen as black spots over light nuclear halos. Magnification is shown by white bars.
that the MAR elements flanking the region under study are located on the nuclear matrix while the whole internal part (between MAR3 and MAR4) is looped out into the nuclear halo. The size of the observed loops seems to vary in different samples probably due to unpredictable folding of the looped fragments in the course of preparation of nuclear halos and also due to variations in loop unfolding in individual nuclear halos. The dimensions of the loop crown usually vary to a large extent in different nuclear halos within the same sample. It is difficult to achieve complete unfolding of DNA loops, some of which still remain supercoiled in nuclear halos. That is why the contour length of the loops observed on such preparations usually constitutes about half of that expected for fully extended DNA (8).

**Induction of differentiation in HL60 cells and analysis of the spatial organization of the DNA region under study in differentiated cells**

HL60 cells are known to undergo lymphoid differentiation in response to DMSO treatment. This differentiation results in the arrest of cell proliferation and in a radical change in the pattern of gene expression (18,19). In particular, the levels of expression of CD22 and GRP43 genes increase drastically upon differentiation of HL60 cells (see below). In order to find out whether the induction of active transcription in the DNA loop under study is accompanied by changes in loop folding, experiments on hybridization with nuclear halos prepared from induced HL60 cells were performed. The same probes as in the experiment with non-induced cells were used. The results of hybridizations are shown in Figure 3. No extended loops were observed in this set of experiments. All hybridization signals were located on the nuclear matrix. This was especially evident while examining microscopic slides with immobilized nuclear halos at low magnification, when a number of nuclear halos could be simultaneously seen (Figure 3, D and D′). When the same samples were examined at high magnification, one or several small loops extending from the central spot were occasionally seen (Figure 3, A′ and A″). A conclusion was made that the induction of active transcription in the DNA loop causes an association of the loop DNA with the nuclear matrix. To verify whether DMSO-induced differentiation of HL60 cells affects the expression of CD22 and GPR43 genes, the levels of the corresponding

![Figure 3. Visualization of the region under study on nuclear halos prepared from DMSO-induced HL60 cells. Panels A–D show DAPI staining of nuclear halos and the results of hybridization are shown in panels A′–D′. Panels A″–D″ show superposition of DAPI staining and hybridization signals, which are seen as black spots over light nuclear halos. Magnification is shown by white bars.](https://academic.oup.com/nar/article-abstract/33/13/4157/1094594)
mRNAs in non-induced and induced cells were estimated by RT–PCR. The results shown in Figure 4 demonstrate that the expression of both genes significantly (at least 10-folds) increases upon differentiation of HL60 cells. Differentiation of HL60 cells results not only in changes in the transcription pattern but also in the arrest of proliferation. Thus, changes in the spatial organization of the genome may be caused also by the arrest of proliferation. To check this possibility, we repeated experiments with non-differentiated HL60 cells incubated for 3 h in the presence of 3\(\mu\)M aphidicolin, a potent inhibitor of DNA polymerases \(\alpha\) and \(\delta\). According to literature data, incubation of proliferating cells with aphidicolin under the above-mentioned conditions causes an almost complete arrest of replication (20). Nevertheless, the nuclear halos obtained from aphidicolin-treated HL60 cells were virtually the same as the nuclear halos obtained from non-treated HL60 cells. Furthermore, the DNA region under study was looped out from the nuclear matrix (data not shown).

Analysis of accessibility of one of the MAR elements flanking the DNA loop at the downstream end for the cleavage by endogenous topoisomerase II

It was demonstrated previously that DNA loop anchorage regions are preferentially accessible for topoisomerase II-mediated cleavage both in high salt-extracted nuclei and in living cells (21–23). Accessibility for topoisomerase II-mediated cleavage can thus be considered as an indication of the attachment of the DNA loop to the nuclear matrix. To verify the supposition that the MAR elements flanking the DNA region under study are involved in the anchorage of the DNA loop to the nuclear matrix in non-differentiated HL60 cells, we studied the accessibility of one of them, MAR4 (see the map in Figure 1), for topoisomerase II cleavage in living cells. The previously described PCR-stop approach was used to follow the progression of topoisomerase II-mediated cleavage. Two DNA fragments of almost the same length (3.4 and 3.7 kb) were selected, one of them (3.4 kb) being located it the middle of the DNA loop and the other (3.7 kb) covering MAR4 and the flanking sequences (see the map in Figure 1). DNA samples were isolated from control cells and from cells treated with 50\(\mu\)g/ml VM26, an inhibitor of topoisomerase II (24), for 2 or 5 h as indicated in the legend for Figure 5. The results shown in Figure 5 demonstrate that DNA samples from both treated and untreated cells served equally well as templates for PCR amplification of the DNA fragment derived from the middle of the loop. In contrast, when the DNA fragment bearing MAR4 was PCR-amplified on DNA templates from VM-26-treated cells, a much weaker signal was observed as compared with that observed upon amplification of the same fragment on a DNA template from non-treated cells. Thus the DNA fragment containing MAR4 is more preferentially cleaved by endogenous topoisomerase II as compared with the fragment derived from the middle of the DNA loop.

DISCUSSION

The results of this study strongly support the model postulating the existence of constitutive and transient associations of DNA with the nuclear matrix (13,14). We have characterized the spatial organization of an \(~170\) kb region of human chromosome 19 and demonstrated that in proliferating HL60 cells this DNA region forms a single loop attached to the nuclear matrix most probably via the flanking MAR elements. As demonstrated using the PCR-stop technique (25), MAR4 is preferentially accessible for topoisomerase II-mediated cleavage \textit{in vivo}. This observation supports the idea that this MAR is indeed associated with the nuclear matrix, as previously it was shown that DNA loop anchorage regions constitute major targets for topoisomerase II-mediated cleavage, both in living
cells and in high salt extracted nuclei (21,23). Although MAR2 and MAR3 present on the left flank of the region under study are located in the MAG gene encoding myelin-associated glycoprotein, the apparent attachment of this region to the nuclear matrix in non-differentiated HL60 cells is not related to transcription as the MAG gene is active only in differentiating oligodendrocytes (26,27). The absence of MAG transcription both in HL60 and DMSO-stimulated HL60 cells was checked by RT–PCR (data not shown).

In differentiated HL60 cells the DNA fragment under study was not looped out from the nuclear matrix. In most of the examined cells the hybridization signals were concentrated in two spots located within the nuclear matrix. Occasionally, very small DNA loops extending from those spots were observed. In other words, the same 170 kb DNA fragment was demonstrated to be looped out from the nuclear matrix in non-differentiated HL60 cells and to be closely associated with the nuclear matrix after DMSO-induced differentiation of these cells. The dramatic change in the spatial organization of the DNA region under study is related with the activation of transcription of CD22 and GPR43 genes in differentiated cells. Thus, the activation of transcription within the DNA loop attached to the nuclear matrix via MAR elements is likely to cause relocation of the whole looped DNA fragment to the nuclear matrix. This interpretation is in perfect agreement with old (28–32) and more recent (7,33) observations demonstrating that transcribed DNA regions become associated with the nuclear matrix. At first glance the above argumentation might be compromised by the fact that CD22 and GPR43 genes are transcribed, although at a very low level, in non-differentiated HL60 cells. However, it seems obvious that in a population of proliferating HL60 cells there is a small fraction of spontaneously differentiated cells. In this case it is quite possible that in all other cells constituting the bulk of the population the CD22 and GPR43 genes are not transcribed at all. An important advantage of our experimental model as compared with that used by Ratsch and collaborators (7) is that in our experiments the configuration of the same genomic region was studied before and after the activation of transcription. In contrast, Ratsch and collaborators (7) compared normal and rearranged c-myc gene loci. They did not study the distribution of MAR elements in normal and in rearranged loci. Although in their study a 20 kb long attachment area was found at a distance of 20 kb downstream to the c-myc gene (in LY66 cells), the transcriptional status of this particular area was not analyzed. Thus, it is difficult to say whether this area represents a constitutive attachment region. Heng and collaborators (33) studied the topological organization of the 40 kb long human protamin gene locus inserted (in some cases as multiple copies) into the mouse genome. In preliminary experiments it was demonstrated that the human protamin gene locus contained one bona fide MAR element. When the genome of transgenic mice contained multiple copies of the above-mentioned fragment of human DNA, only some MARs were actually fixed at the nuclear matrix. Again, a positive correlation between the association with the nuclear matrix and transcription of protamin genes was observed. On the basis of these and other observations the authors suggested that within a loop formed by constitutive attachment sites there could be facultative MAR elements that ensure the attachment of active genes to the nuclear matrix (33). Alternatively, active genes may be transiently attached to the nuclear matrix via matrix-bound transcriptional complexes (34). The resolution of our present experiments as well as the resolution of experiments of Heng and collaborators (33) does not permit a choice between the two above-mentioned possibilities to be made. Nevertheless, it is worth mentioning that in our previous studies we did not find any additional MAR elements within the domain framed by MARs 3 and 4 (Figure 1). Thus it seems more likely that active genes are transiently attached to the nuclear matrix via transcriptional complexes.

Differentiated HL60 cells stop proliferation. Thus, the possibility should be considered that it is the proliferation arrest and not changes in the transcription pattern that causes spatial reorganization of the genome, including the collapse on the nuclear matrix of the DNA loop under study. However, in this case the spatial reorganization of the genome must be global as all DNA is replicated in non-induced HL60 cells. This global spatial reorganization of the genome is likely to affect the shape and/or stability of nuclear halos. We detected no differences in nuclear halos obtained from proliferating HL60 cells and HL60 cells treated with a replication inhibitor, aphidicolin. Furthermore, the arrest of replication by aphidicolin did not cause a collapse of the DNA loop under study on the nuclear matrix. Thus it is probable that the association of this DNA loop with the nuclear matrix is indeed caused by induction of active transcription within this particular DNA loop in differentiated HL60 cells.

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