Visualization of individual DNA loops and a map of loop domains in the human dystrophin gene

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ABSTRACT

The organization of the human dystrophin gene into loop domains has been studied using two different experimental approaches: excision of DNA loops mediated by nuclear matrix-bound topoisomerase II and in situ hybridization of different probes with histone-depleted nuclei (nuclear halos). Our objective was to examine if the DNA loops mapped by this biochemical approach coincide with loops visualized by microscopy. The results obtained using both approaches were in good agreement. Eight loops separated by attachment regions of different length were mapped in the upstream part (up to exon 54) of the gene by topoisomerase II-mediated excision. One of these loops was then directly visualized by in situ hybridization of the corresponding bacmid clone with nuclear halos. This is the first direct demonstration that a DNA domain mapped as a loop using a biochemical approach corresponds to a loop visible on cytological preparations. The validity of this result and of the whole map of loop domains was confirmed by in situ hybridization using probes derived from other attachment regions or loops mapped by topoisomerase II-mediated cleavage; these probes hybridized on the core or halo region, respectively, of nuclear halos. Our results demonstrate that a single transcription unit may be organized into several loops and that DNA loop attachment regions may be fairly long. Three out of four replication origins mapped in this gene co-localize with loop attachment regions, and the major deletion hot spot is harbored in an attachment region. These results strongly suggest that partitioning of genomic DNA into specific loops attached to a skeletal structure is a characteristic feature of eukaryotic chromosome organization in interphase.

INTRODUCTION

It has become increasingly evident that epigenetic factors play an important role in eukaryotic genome functioning. As well as DNA methylation and modifications of histones (1), the mechanisms which determine specific positioning of individual DNA sequences within nuclei can also be regarded as a part of epigenetic regulatory systems. Folding of DNA into a nucleosomal fiber may bring together relatively distant protein binding sites (2) and higher-order chromatin folding may bring together regulatory sequences separated by dozens of kilobases of DNA and thus favor the assembly of active chromatin hubs (3). To understand better the mechanisms of these long-distance interactions of regulatory elements it is necessary to clarify the basic principles of DNA packaging in interphase chromosomes, especially at higher levels of DNA compaction. At present, our knowledge about these levels of DNA spatial organization in the nucleus is rather schematic and far from complete. According to the radial loop model of eukaryotic chromosomes the chain of nucleosomes is packed into a 30 nm-diameter fibril which is organized into large loops attached to a scaffold or matrix (4–7), and although this model has been discussed in the literature over the last 25 years it still remains neither proven nor disproved [for a review see Heng et al. (8)]. Several approaches were developed to map the bases of DNA loops [for reviews see Razin et al. (9) and Mirkovitch et al. (10)]. Nuclear scaffold or matrix attachment regions [SARs (11) or MARs (12)] are frequently considered as DNA loop attachment sequences; however, experimental data questioning this supposition have been presented (13). We have developed a protocol for mapping DNA loop attachment regions which is based on excision of loops mediated by nuclear matrix-bound topoisomerase II (14–16). The attachment regions mapped by the topoisomerase II-mediated DNA loop excision procedure do not necessarily contain MARs/SARs (16). In addition, many MARs/SARs are present in DNA loops mapped by the above protocol [i.e. outside attachment regions (17)]. Although there are strong arguments supporting validity of the topoisomerase II-mediated DNA loop excision protocol, it has not yet been established whether DNA loops mapped using this protocol correspond to those seen under the microscope. This is also

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true for all other biochemical protocols used so far for mapping loops.

The objective of the present work was to compare directly the DNA loops mapped by our biochemical approach with those visualized by microscopy. We present a domain map of an extended genomic area which includes the major part of the human dystrophin gene, and show for the first time that DNA loops mapped by topoisomerase II-mediated excision do correspond to the loops that can be visualized by hybridization of specific probes with nuclear halos. We also show that a single transcription unit may be organized into several DNA loops. Furthermore, we found that the major deletion hot spot in the dystrophin gene is located in one of the loop attachment regions.

**MATERIALS AND METHODS**

**Cell culture**

Human erythroleukemia cells HEL 92.1.7 (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum.

**Mapping of DNA loop attachment regions using topoisomerase II-mediated DNA loop excision**

The protocol for topoisomerase II-mediated DNA loop excision was described previously (16,17). Briefly, cells were collected by centrifugation and embedded into blocks of 1.5% low-melting agarose prepared in PBS buffer. To permeabilize cells and extract them with 2 M NaCl, blocks with embedded cells were incubated in a buffer containing 0.2% Nonidet P-40 (NP-40), 2 M NaCl, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 20 mM Tris–HCl (pH 7.5) for 1 h at 4°C. The blocks were then washed three times (30 min each) at 4°C in a buffer containing 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 10 mM MgCl₂. After washing, they were placed in the same 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]. The slides were then sequentially washed (1 min each wash) 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.

**Hybridization in situ, immunolabeling and microscopy**

Nuclear halos were treated sequentially with RNase A (100 μg/ml in 2× 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, 2× SSC for 5 min at 74°C, dehydrated in cold 70, 80 and 96% ethanol and air-dried.

Most probes for in situ hybridization were prepared by PCR using the primer pairs listed in Table 1 and labeled with [α-32P]dCTP using Megaprime kits (Amersham). 

**Preparation of nuclear halos**

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 PMSF, 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 pelleted onto silane-coated microscope slides using a Cytospin centrifuge. The cells on the slides were then treated (4 min at 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]. The slides were then sequentially washed (1 min each wash) 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.

**Table 1. Primers for PCR-derived probes to detect restriction fragments on PFGE gels**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer pairs</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC, right end (exon 11)</td>
<td>5′-GTACATGATGGATTGACAGC-3′</td>
<td>165</td>
</tr>
<tr>
<td>CD, right end (exon 29)</td>
<td>5′-CATGCTAGCTACCTGAGG-3′</td>
<td>153</td>
</tr>
<tr>
<td>DE, right end (exon 41)</td>
<td>5′-ATATTAGGAAATTGATCGG-3′</td>
<td>144</td>
</tr>
<tr>
<td>EF, left end (exon 43)</td>
<td>5′-GCCGAGTAAACACCTCAGTC-3′</td>
<td>175</td>
</tr>
<tr>
<td>FG, right end (exon 54)</td>
<td>5′-TTTCAAGTTCTAGGGATGAA-3′</td>
<td>165</td>
</tr>
</tbody>
</table>

Hybridization probes for indirect end-labeling were prepared by PCR using the primer pairs listed in Table 1 and labeled with [α-32P]dCTP using Megaprime kits (Amersham).
samples were washed twice in 50% formamide, 2°C anti-goat and goat anti-chicken) both conjugated with Alexa 647 were carried out, two additional layers of antibodies (chicken biotinylated BAC 662G16 DNA or fragments of this insert antibodies (Molecular Probes). When hybridization with using an Alexa 488 signal amplification kit for mouse monoclonal antibodies conjugated with Alexa 488 was followed by incubation at 37°C for 2 h. Hybridization was carried out overnight at 40–45°C. After hybridization the samples were washed twice in 50% formamide, 2× SSC at 43–48°C for 20 min.

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 (Molecular Probes). When hybridization with biotinylated BAC 662G16 DNA or fragments of this insert were carried out, two additional layers of antibodies (chicken anti-goat and goat anti-chicken) both conjugated with Alexa 488 were used. DNA was stained with DAPI (4’,6-diamidino-2-phenylindole). The results were examined under a fluorescence microscope (Axioplan Opton) and recorded using a cooled CCD camera (AT200, Photometrics, Tucson, AZ). The contour length of DNA loops on images was measured using the program Scion Image, version 4 beta.

## RESULTS

### Mapping the organization of the human dystrophin gene into DNA loop domains by topoisomerase II-mediated loop excision

The human dystrophin gene spans almost 2.5 Mb on the short arm of the X chromosome (Xp21) (19,20). In female cells one copy of the X chromosome is inactive, and it is not clear whether the organization of the dystrophin gene into loop domains in active and inactive copies is identical. In order to facilitate the interpretation of the results we therefore used HEL 92.1.7 cells derived from a male patient. We first studied the partitioning of the dystrophin gene into loop domains using the topoisomerase II-mediated DNA loop excision method (15,16,18). The principle of this method is that in high salt-extracted nuclei only the DNA loop attachment sites constitute targets for cleavage by topoisomerase II of the nuclear matrix/chromosomal scaffold (21,22). Permeabilized cells are extracted with 2 M NaCl and incubated with the specific topoisomerase II religation inhibitor VM-26, which traps this enzyme at its sites of action and causes DNA cleavage upon deproteinization (15–18). The sites of cleavage are deduced from the length of truncated restriction fragments determined by indirect end-labeling. DNA between the cleavage regions is thought to be looped out (15,16). Importantly, it has been demonstrated that the same genomic regions constitute major targets for topoisomerase II-mediated cleavage both in high salt-extracted nuclei and in living cells (15,16), suggesting strongly that the sites of DNA contact with the nuclear matrix that can be mapped in high salt-extracted nuclei pre-exist in living cells. It is also of importance that attachment regions mapped by this protocol do not necessarily correlate with transcribed regions; thus in the ribosomal gene repeat of Chinese hamster ovary cells the single attachment region is localized in the non-transcribed spacer (15), and hence cannot represent an area of DNA contact with the nuclear matrix that originates as an artefact due to precipitation of transcription complexes in the course of high-salt extraction (15).

In the present study, DNA loop attachment regions were mapped relative to SfiI restriction sites using DNA fragments separated by PFGE, as described previously (16,17,23) (Fig. 1). Most of the hybridization probes were PCR-derived fragments representing exons located close to the SfiI sites, and fragment AB was probed with the insert of a λ phage recombinant clone covering SfiI site B (24). Results of mapping experiments are shown in Figure 1. Some of the attachment regions in the dystrophin gene, notably attachment regions 3, 5 and 8, appeared to extend over more than a hundred kb as judged by the length heterogeneity of the truncated fragments; we believe that this heterogeneity reflects inactivity that induce complete cleavage by topoisomerase II within a long attachment region (see Fig. 1C, right scheme), because the fragment lengths become more homogeneous as the concentration of VM-26 increases (Fig. 1) (see Discussion). This was expected from previous results and seems to be a characteristic feature of DNA loops in AT-rich isochromes (25). Two observations argue against the suggestion that areas of distributed hybridization simply represent smears originating as a result of non-specific DNA degradation. First, the sizes of extended hybridizing areas vary depending on the probe used. At the same time, in no case does the distribution of hybridizing material in wide areas reflect distribution of DNA as revealed by ethidium bromide staining (Fig. 1B). Thus it is more likely that areas of distributed hybridization reflect the existence of extremely long attachment regions, as shown in the scheme in Figure 1C (right side). It may be of interest that a reasonably good correlation between the positions of attachment regions and recombination-prone regions in the dystrophin gene was observed. Thus, attachment region 5 harbors the major deletion hot spot of the gene (26).

Although the identity of the target regions for topoisomerase II-mediated DNA cleavage in living cells and in permeabilized high-salt extracted cells has been established

### Table 2. Primers for PCR-derived probes for in situ hybridization

<table>
<thead>
<tr>
<th>Probe for</th>
<th>Primer pairs</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop 1</td>
<td>5’-GCGTAAAAGGTTTCAATAGAGG-3’</td>
<td>2019</td>
</tr>
<tr>
<td></td>
<td>3’-AAAGTGCCTGATGAAATACC-5’</td>
<td></td>
</tr>
<tr>
<td>AR 3</td>
<td>5’-AATAATTGCGATTTCTTCCGCTAG-3’</td>
<td>2334</td>
</tr>
<tr>
<td></td>
<td>3’-ATTAGAAGGGAGACCTTTCAA-5’</td>
<td></td>
</tr>
<tr>
<td>Loop 5</td>
<td>5’-GGGAATGCTCGTCGTTGTTTCT-3’</td>
<td>2749</td>
</tr>
<tr>
<td></td>
<td>3’-CGGACTCCGTAGGTTTCTAAAAT-5’</td>
<td></td>
</tr>
<tr>
<td>AR 4</td>
<td>5’-ATTAAATCCTAATGTCAGGCTTCAG-3’</td>
<td>4125</td>
</tr>
<tr>
<td></td>
<td>3’-AAATGATAGCCCCTGCATAGACAG-5’</td>
<td></td>
</tr>
<tr>
<td>Loop 7</td>
<td>5’-TGTTTTGAATGAGAGAAAGGCA-3’</td>
<td>5012</td>
</tr>
<tr>
<td></td>
<td>3’-GGACCCATTAAATGGGACTTAGAAAT-5’</td>
<td></td>
</tr>
<tr>
<td>AR 7</td>
<td>5’-ACTATAACCATATAGCGAATGCC-3’</td>
<td>2118</td>
</tr>
<tr>
<td></td>
<td>3’-GATAAGTAGCGCGATGCTAT-5’</td>
<td></td>
</tr>
<tr>
<td>Loop 8</td>
<td>5’-AAAGATGAGCTTTTCCTAGTGAT-3’</td>
<td>3858</td>
</tr>
<tr>
<td></td>
<td>3’-AGTAGAAGGTTATACCAGGGCA-5’</td>
<td></td>
</tr>
<tr>
<td>AR 8</td>
<td>5’-ATTGTGGAAAAATGTTAGGCCCTGCA-3’</td>
<td>3779</td>
</tr>
<tr>
<td></td>
<td>3’-TATACAAATCTGGTGCTCCCTCAA-5’</td>
<td></td>
</tr>
</tbody>
</table>

AR, attachment region.
Visualization by \textit{in situ} hybridization of DNA sequences located in loops or loop attachment regions

Although the protocol for mapping attachment regions by topoisomerase II-mediated loop excision is supported by much evidence (15), it was not yet demonstrated directly that DNA regions mapped as loops using this protocol are identical to those that are seen by microscopy to be looped out in high-salt-extracted nuclei. To examine this correlation we hybridized specific probes from the loop and attachment regions observed in Figure 1 with nuclear halos, which represent the crown of extended DNA loops fixed at the nuclear matrix and visible by microscopy (27–31). The dimensions of halos have been used widely as a simple experimental approach to estimate the sizes of DNA loops (27,28), and visualization of specific DNA sequences on halos by hybridization opens the possibility to study their partitioning between matrix-bound and looped-out DNA fractions (29–31).

Nuclear halos were prepared as described (30) with slight modifications (see Materials and Methods). Their size depends on the concentration of Cu ++ ions in the buffer at the step of histone removal, and we carried out a set of preliminary experiments aimed to find the most reproducible conditions for preparation of halos from HEL cells. Three parameters were taken into consideration: the preservation of the central core region as inspected after immunostaining of lamin A (Fig. 2A and B), the average radius of the halo as measured after staining with DAPI, and the distance at which the X chromosome-specific alphoid satellite (alphX) was extended from the core. Within certain limits of Cu ++ concentration the ratio (radius of alphX/radius of halo) was reasonably constant (0.493 ± 0.047, n = 139) and we selected conditions (0.1 mM Cu ++) permitting maximal halos to be observed while the above ratio was kept constant. Typical images of halos and signals from the alphX probe are shown in Figure 2.

Short PCR-derived probes representing either loops or attachment regions as observed in Figure 1 were then hybridized with halo preparations and signals over the core or halo region were quantitated. DNA loops could fold over the core region when the three-dimensional nuclear matrix or halo region were quantitated. DNA loops could fold over the core but probes for loop DNA could appear over the halo. We inspected ~100 halos for each probe, and signals were considered as showing attachment regions when ~90% were over the core region or loops when >75% were over the halo region; intermediate signal distributions were not observed (Table 3). A probe from attachment area 8 produced a single signal located almost exclusively within the core region, as predicted (Fig. 3A and A’). In contrast, a short probe from the central part of loop 7 produced signals localized for the most part in the halo far from the core (Fig. 3B and B’); hybridization within the core was detected in only 19% of the nuclei and was obviously due to the unfavorable folding of DNA loops, as discussed above. The difference in hybridization patterns of loop and attachment region probes is also clearly seen at low magnification when several nuclei can be inspected simultaneously (Fig. 3C and D). Short probes for

previously in several models, we confirmed this conclusion for the region FG that was of particular interest for the present study. The results in the insert in Figure 1B show that in this case also, the patterns of topoisomerase II-mediated DNA cleavage in living cells and in high-salt-extracted cells are essentially identical. The rationales for this similarity have been discussed previously along with other arguments for the validity of this method (15,16).
several other attachment regions or loops mapped by topoisomerase II-mediated cleavage hybridized to cores or halos, respectively (Fig. 4 and Table 3). We conclude from the data summarized in Table 3 that in situ hybridization on nuclear halos confirms the identification of loop or attachment region DNA as defined by topoisomerase II-mediated loop excision.

Visualization of an individual DNA loop in the dystrophin gene

With the aim of visualizing an individual loop, we used as probe a BAC clone which contains an insert of ~220 kb covering the predicted DNA loop between attachment regions 7 and 8 (Fig. 1). When this probe was hybridized in the absence of Cot1 DNA, an intense labeling of the whole halo was observed (Fig. 5A') as predicted because the insert in this BAC contains a large number of repetitive elements. The signals originating from these repetitive elements were efficiently competed when a 100–500× excess (w/w) of human Cot1 DNA was added to the reaction mixture.

In these conditions, single DNA loops became clearly visible; representative images are shown in Figure 5B'–E'. Such bona fide U-shaped loops were seen in 14% of 200 nuclei inspected, and broken loops in 47% (Fig. 5F'). In 28% of the nuclei the signals were distributed in a disordered fashion over both the core and halo (for example, the right nucleus in Fig. 5C'), which is likely to result from distortion of the halo in the course of preparation and/or from an unfavorable loop position on the plane surface of the slide. No signals were detected in 11% of the nuclei, and this percentage was much higher when short probes were used (Table 3). We conclude that the DNA loops visualized in Figure 5B'–E' represent the loop flanked by attachment regions 7 and 8 as mapped by topoisomerase II-mediated cleavage (Fig. 1).

The average length of 20 clearly identifiable loops, measured by tracing their contour as shown in Figure 5B"–E", was 65.0 μm with a SD of 20.3 μm. The length of a fully extended DNA loop of 220 kb should be ~65 μm, and our measured length fits this value perfectly. Other researchers have reported that the apparent length of DNA in nuclear halos is only 50–75% of that of fully extended DNA (30); the difference between these results and ours may be due to the fact that only short stretches of DNA (15 kb) were measured in previous work while we measured a length of 220 kb.

DISCUSSION

The results presented here clearly demonstrate (i) the specificity of the organization of genomic DNA into loops and (ii) the validity of the protocol for mapping loops by topoisomerase II-mediated excision. The DNA loop in the dystrophin gene mapped using this protocol could be visualized directly by in situ hybridization with nuclear halos. The validity of the map of the spatial organization of this gene

### Table 3. Distribution of hybridization signals over nuclear cores or loop halos

<table>
<thead>
<tr>
<th>Probe</th>
<th>Number of nuclei</th>
<th>% of signals over core</th>
<th>% of signals over halo</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR 8</td>
<td>100</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>Loop 7</td>
<td>100</td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>AR 7</td>
<td>120</td>
<td>89</td>
<td>11</td>
</tr>
<tr>
<td>AR 5</td>
<td>90</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>AR 4</td>
<td>100</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>Loop 3</td>
<td>140</td>
<td>23</td>
<td>77</td>
</tr>
<tr>
<td>AR 3</td>
<td>100</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>Loop 1</td>
<td>120</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

Nuclear halos were hybridized with the probes shown in Table 2 which detect loops or ARs as mapped in Figure 1. Only nuclei showing a hybridization signal (~65% of the total) were scored.
constructed using topoisomerase II-mediated loop excision was confirmed using *in situ* hybridization with probes representing other attachment regions and loops. Although individual DNA loops extending from the nuclear matrix or scaffold were observed in previous investigations (31,32), no attempt had been made before now to correlate these data with the results of loop mapping by biochemical approaches. Furthermore, although it has been proposed that the specificity of DNA organization into loops in interphase chromosomes is flexible and simply reflects partitioning of the genome into transcribed and non-transcribed regions (31), the dystrophin gene is not transcribed in the erythroid cells used in our study (data not shown) and hence, at least in this case, mechanisms that are not directly related to transcription are responsible for organization and maintaining of DNA loop attachment sites. The lengths of loop attachment regions 3, 5 and 8, which is >100 kb as seen by the breadth of the hybridizing bands of fragments truncated by topoisomerase II cleavage (Fig. 1), are at first view unexpectedly large. We believe that this apparent
length reflects the existence of long regions of DNA attachment to the nuclear matrix and inability to obtain complete cleavage by the enzyme, because the length distribution of these fragments becomes more homogeneous as the concentration of VM-26 increases. Similar incomplete cleavage has been observed in other regions (23,25). Alternatively, the cell population could be heterogeneous with different attachment sites being selected from a region of potential attachment in different cells and/or at different times in the cell cycle. It should be mentioned, however, that the existence of long attachment regions is in perfect agreement with the intense DAPI staining of the core region of nuclear halos (nuclear matrix) observed by us and by others [see, for example, figures 1 and 3 in Gerdes et al. (30)].

The map of dystrophin gene organization into loop domains presented in this paper is interesting from several points of view. It demonstrates clearly that a single transcription unit may be organized into several loops and thus that the attachment regions do not constitute barriers for transcription. It is also of interest that three out of four replication origins previously mapped in this gene (33) co-localize with loop attachment regions 3, 5 and 7 (Fig. 1) within the limits of resolution of the corresponding methods. Loop attachment region 5 harbors the major deletion hot spot of the dystrophin gene (26,34). In this context it may be of interest that a recombination hot spot in the vicinity of the Chinese hamster gene GNA3 (also in an AT-rich isochore) was found to reside in a loop attachment region (25). Our findings may eventually help to explain the nature of recombination hot spots in the dystrophin gene. In particular, the possibility that preferential recombination between MARs results in the loss of individual loops (35) deserves further analysis.

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