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Mapping of the nuclear matrix-bound chromatin hubs by a new M3C experimental procedure

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ABSTRACT

We have developed an experimental procedure to analyze the spatial proximity of nuclear matrix-bound DNA fragments. This protocol, referred to as Matrix 3C (M3C), includes a high salt extraction of nuclei, the removal of distal parts of unfolded DNA loops using restriction enzyme treatment, ligation of the nuclear matrix-bound DNA fragments and a subsequent analysis of ligation frequencies. Using the M3C procedure, we have demonstrated that CpG islands of at least three housekeeping genes that surround the chicken α-globin gene domain are assembled into a complex (presumably, a transcription factory) that is stabilized by the nuclear matrix in both erythroid and non-erythroid cells. In erythroid cells, the regulatory elements of the α-globin genes are attracted to this complex to form a new assembly: an active chromatin hub that is linked to the pre-existing transcription factory. The erythroid-specific part of the assembly is removed by high salt extraction. Based on these observations, we propose that mixed transcription factories that mediate the transcription of both housekeeping and tissue-specific genes are composed of a permanent compartment containing integrated into the nuclear matrix promoters of housekeeping genes and a 'guest' compartment where promoters and regulatory elements of tissue-specific genes can be temporarily recruited.

INTRODUCTION

The current model for the control of eukaryotic gene expression by multiple regulatory elements suggests that these regulatory elements (e.g. enhancers and promoters) are assembled into a single activating complex called the 'active chromatin hub'. The existence of such activating complexes had been anticipated but was experimentally proven only recently when the chromosome conformation capture (3C) experimental approach was developed (1,2). Using this method, it was demonstrated that distant regulatory elements of the mouse β-globin gene domain form a common activating complex with the promoters of active globin genes (3–5). Similar observations were made for other genomic domains (6–9). Our goal was to determine how active chromatin hubs are incorporated into the system of nuclear compartmentalization. With this aim in mind, we analyzed the possible role of the nuclear matrix in active chromatin hub organization.

The nuclear matrix is an operationally defined skeletal structure that underlies the nucleus (10). Although the nature of the nuclear matrix is still unclear and its existence has been questioned (11–13), it is likely that nuclear compartmentalization requires an underlying skeletal structure of some kind (14–17). For this reason, the notion of the nuclear matrix, or 'nuclear skeleton', has been maintained over the last 35 years. The nuclear matrix can be isolated by high salt extraction of nuclei that are pretreated with nucleases (10). A fraction of DNA remains associated with the nuclear matrix after high salt extraction. This DNA is usually referred to as 'nuclear matrix DNA' or 'nmDNA', and the sizes of nmDNA fragments depend on the intensity of the nuclease treatment (18). Numerous reports indicate that different regulatory elements (e.g. promoters, enhancers and insulators) are associated with the nuclear matrix (19–22). Therefore, it is reasonable to hypothesize that active chromatin hubs are assembled on the nuclear matrix. If this is true, then DNA sequences that are involved in the formation of an active chromatin hub should remain associated with the nuclear matrix in close proximity to each other, following the removal of the major chromatin fraction by nuclease treatment and

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high salt extraction. To test this model, we have modified the 3C approach to analyze the spatial proximity of nuclear matrix-bound DNA fragments. One of the critical steps in the standard 3C procedure is the fixation of DNA:protein complexes with formaldehyde. This step is necessary to preserve the integrity of the DNA:protein complexes during the subsequent lysis of nuclei with sodium dodecyl sulfate (SDS). The complexes of DNA that are associated with the nuclear matrix are resistant to high salt extraction in the absence of formaldehyde fixation. Concurrently, this extraction removes histones and improves the accessibility of the DNA to restriction enzymes. In our protocol, we isolated the so-called nucleoids (high salt-extracted nuclei that contain all genomic DNA organized into loops bound to the nuclear matrix) (23), removed the distal segments of the DNA loops by treatment with restriction enzymes (24) and then analyzed the frequencies of ligation of different nuclear matrix-bound DNA fragments. We applied this experimental approach, Matrix 3C (M3C), to study a role of the nuclear matrix in the spatial organization of a fragment of chicken chromosome 14 that includes the α-globin gene domain. Using this model, we have demonstrated that, across cells of different lineages, neighboring CpG islands bearing housekeeping gene promoters form high salt-resistant complexes on the nuclear matrix. In erythroid cells, erythroid-specific regulatory elements are recruited to these complexes. However, the interaction of erythroid-specific regulatory elements, including the *alpha-D* globin gene (α^D gene) promoter, with the nuclear matrix-bound promoters of housekeeping genes, is not resistant to high salt extraction.

MATERIALS AND METHODS

Cell culture

The avian erythroblastosis virus-transformed chicken erythroblast cell line HD3 [clone A6 of line LSCC (25,26)] and the DT40 lymphoid cell line (CRL-2111, ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 2% chicken serum and 8% fetal bovine serum at 37°C with 5% CO_2. The medium for the DT40 cells was supplemented with 50 μM of β-mercaptoethanol. The induction of HD3 cells to the terminal erythroid state was performed according to previously described protocols (6)

Isolation of nuclear matrices for M3C analysis, and ligation of nuclear matrix-bound DNA fragments

Cell nuclei were prepared according to protocols described elsewhere (27). To extract histones, 10^8 nuclei were suspended in 10 ml of ice-cold buffer A [10 mM PIPES (pH 7.8), 100 mM NaCl, 0.3 M Sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 0.1 mM PMSF]. An equal volume of buffer A supplemented with 4 M NaCl was slowly added to the suspension with gentle mixing. After a 30-min incubation on ice, the nucleoids were precipitated (10 min, 3200 *g*) and washed three times by resuspension–precipitation with a restriction buffer [50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT, 0.1 mM PMSF]. After washing, the nucleoids were resuspended in 10 ml of restriction buffer supplemented with BSA (100 μg/ml), and 2000 U/ml of each of the restriction nucleases, BamHI and BglII (NEB), were added to the suspension. The suspension was incubated for 1 h at 37°C with gentle mixing. The reaction was terminated by adding 0.5 M EDTA to a final concentration of 20 mM and 4 M NaCl to a final concentration of 2 M. After incubation on ice for 30 min, the nuclear matrices were precipitated (10 min, 3200*g*), washed three times with 10 ml of ligation buffer [40 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM PMSF and 100 μg/ml BSA] and finally resuspended in 1 ml of ligation buffer. T4 DNA ligase (Fermentas) was added to a final concentration of 90 U/ml, and the suspension was incubated for 3 h at 4°C with gentle mixing. The matrices were then precipitated, proteins were digested by proteinase K, and DNA was isolated by a conventional phenol–chloroform extraction procedure. For microscopic analysis, the nuclei, nuclear halos and nuclear matrices were pelleted onto silane-coated microscope slides using a Cytospin centrifuge, immuno-stained with antibodies targeting the lamins A and C (Abcam, USA) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The slides were examined under a fluorescence microscope (Axioplan Opton) and recorded using a cooled CCD camera (AT200, Photometrics, Tucson, AZ, USA). When micrococcal nuclease was used for separation of distal parts of DNA loops, the nuclei were pre-extracted with 0.5 M NaCl solution. With this aim, the suspension of nuclei in buffer A was mixed with an equal volume of buffer A supplemented with 1 M NaCl. After a 30-min incubation on ice, the nuclei were precipitated and washed twice with the buffer containing 10 mM Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 1 mM $CaCl_2$. They were then resuspended in the same buffer at a concentration of 10^9 nuclei per ml and treated with micrococcal nuclease (20–100 U/ml) for 15 min at 37°C. The reaction was terminated by addition of EDTA to a final concentration of 20 mM, and the high salt extraction was carried out as described above.

TaqMan real-time polymerase chain reaction analysis of ligation products

Ligation products were analyzed according to the conventional 3C assay protocol (6,28). Primers and TaqMan probes were designed using the DNA sequence AC172304 (GenBank) and Primer Premier 5 computer software (PRIMER Biosoft International). The sequences of the primers and TaqMan probes are listed in the Supplementary Tables S1 and S2. A random ligation control was generated using DNA from a bacterial artificial chromosome bearing the chicken α-globin gene domain and flanking DNA (clone CH261-75C12, CHORI BACPAC Resources Center). Each real-time polymerase chain reaction (PCR) reaction was prepared in a volume of 20 μl and included 200 ng of a M3C DNA template or 200 ng of digested and re-ligated chicken genomic DNA along with 2, 20, 200 or 2000 pg of the BAC random ligation template, 1× PCR buffer [50 mM Tris (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween 20], 0.5 μM of

each primer, 0.25 μM of a TaqMan probe (5′-FAM dye, inside BHQ-1 quencher), 0.2 mM of each dNTP and 1 U of Hot start Taq DNA polymerase (Sibenzyme). The PCR reaction was performed according to the following protocol: initial denaturation for 5 min at 94°C, 60 cycles of 15 s at 94°C, 60 s at 60°C and plate read. The conventional 3C experiments were carried out as described (6,28).

RESULTS

The workflow for the M3C procedure is shown in Figure 1A. To analyze the association frequencies of nmDNA fragments, it was first necessary to isolate nuclear matrices that contained relatively short fragments of attached DNA and possessed either identical or compatible cohesive ends to allow for subsequent ligation. To satisfy these conditions, we used restriction enzymes to cleave off the distal segments of DNA loops (24). Restriction enzymes do not work efficiently on chromatin templates. Therefore, we first removed histones by performing a high salt (2 M sodium chloride) extraction of the isolated nuclei to isolate the so-called nucleoids (23), which were then treated with the appropriate restriction enzymes. The cleaved-off DNA fragments were removed by washing. Microscopic imaging analyses demonstrated that nuclear halos (DNA loops that are bound to the nuclear matrix) are efficiently removed following the treatment of nucleoids with restriction enzymes and subsequent washing steps (Figure 1B). A detailed description of the procedure that was used for the isolation of nuclear matrices and their bound nmDNA fragments can be found in the 'Materials and Methods' section. The nuclear matrices that contained attached DNA fragments were treated with DNA ligase. They were then lysed, and the DNA was isolated. The frequencies of cross-ligation of different DNA fragments were analyzed using TaqMan Real-Time PCR according to the standard 3C protocol. To test our new experimental approach, we decided to use a fragment of chicken chromosome 14 that contains the α-globin gene domain and surrounding housekeeping genes (Figure 2A). To discriminate the permanent (cell lineage-independent) and erythroid cell-specific interactions, we analyzed the spatial configuration of the gene locus in erythroid (HD3) and lymphoid (DT40) chicken cells in parallel. In some experiments, HD3 cells were induced to terminal erythroid differentiation. In a result of this induction, the expression of the 'adult' alpha-type globin genes was significantly stimulated (6). The combination of BglII and BamHI restriction enzymes was used for DNA cleavage. These enzymes recognize different consensuses (AGATCT and GGATCC) but produce compatible DNA ends which can be cross-ligated. Combined treatment with BamH1 and BglII ensured cleavage of the gene locus being studied into a set of DNA fragments that ranged in size from 0.02 to 7.7 kb. We first analyzed relative representation in the nuclear matrix DNA of DNA fragments bearing important functional elements. With this aim, the test-amplicons were designed within each selected

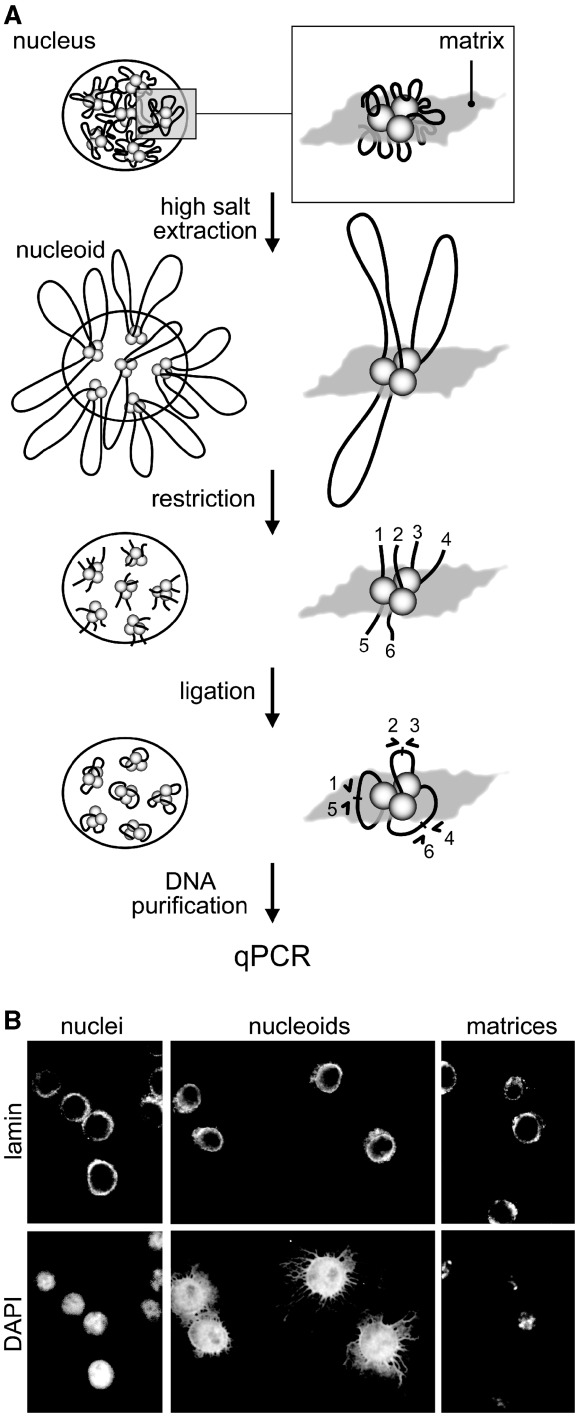

Figure 1. The principle behind the M3C protocol. (**A**) A schematic showing the main steps of the M3C procedure (see the text for explanations). (**B**) Microscope images of nuclei, nucleoids and nuclear matrices from proliferating HD3 cells. In each case, the same confocal section is shown with immunostained lamins A and C (upper row) and DNA stained by DAPI (lower row).

fragment and TaqMan real-time PCR was carried out. The results of these experiments (Supplementary Figure S1A) demonstrated that in both DT40 and HD3 (either proliferating or induced) cells DNA fragments bearing CpG islands of *C16orf35* and *TMEM8* genes

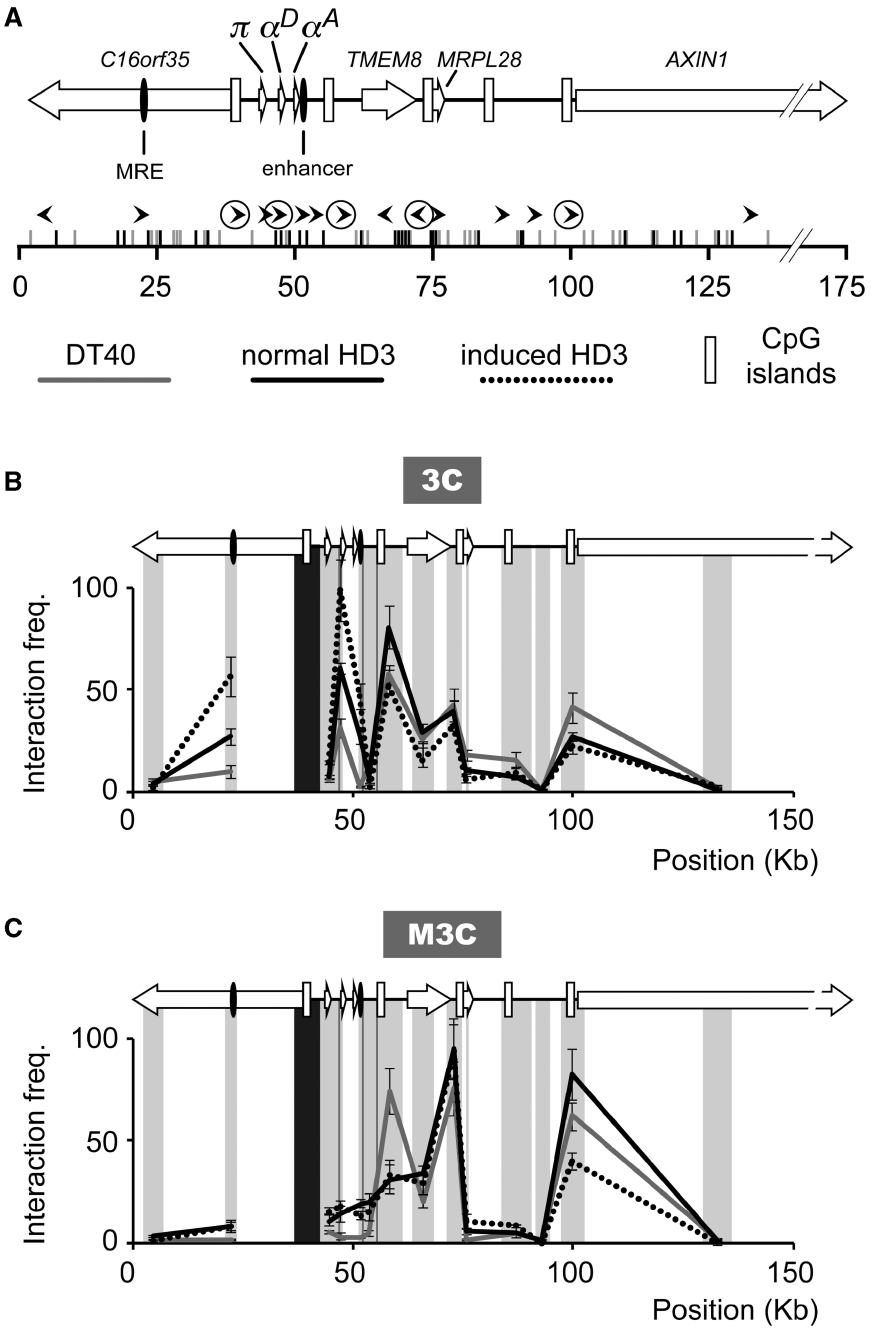

Figure 2. Comparison of the results for the 3C and M3C experiments. (**A**) A schematic showing positions within the gene locus being studied of genes (open rectangles with arrows indicating direction of transcription), CpG islands (vertical open rectangles) and regulatory elements (closed ovals). Positions of Bam HI and BglII restriction sites are shown correspondingly by black and gray vertical lines above the scale. The horizontal tailless arrows show positions and directions of PCR primers. The primers on anchor fragments are encircled. The scale is in kilobase and '0' point of the scale was arbitrarily placed at the 3′-end of the *C16orf35* gene. (**B**) The results of the 3C experiments in which the anchor was placed on the *C16orf35* CpG island. The graphs show the relative frequencies of interactions between the anchor fragment (dark gray shadowing) and upstream and downstream fragments (light shadowing). The borders between neighboring fragments are indicated by dark gray lines. The regions with a white background were not analyzed. The maximal interaction frequency (the amount of the most abundant ligation product that was normalized using 3C data for the ERCC3 gene domain) observed in experiments with the three cell lines studied was arbitrarily considered to be 100, and results were normalized to this value. Error bars represent the SEM for three independent experiments. (**C**) The results of the M3C experiments in which the anchor was placed on the *C16orf35* CpG island. All designations are the same as in (B). The maximal interaction frequency (the amount of the most abundant ligation product) observed for each cell line in this experiment and in experiments with other anchors (Figure 4) was arbitrarily considered to be 100, and results were normalized to this value. Error bars represent the SEM for three independent experiments.

were much more abundant in nuclear matrix DNA than DNA fragments bearing the 3′-end of the *C16orf35* gene, the MRE and the promoter of the α^D gene. We have also prepared nuclear matrix DNA using a limited treatment with micrococcal nuclease to remove the distal parts of DNA loops. The relative abundance in this DNA preparation of fragments bearing the 3′-end of the *C16orf35* gene, the *C16orf35* CpG island and the α^D gene was about the same as in nuclear matrix DNA obtained using combined treatment with BamHI and BglII restriction enzymes (Supplementary Figure S1B).

We have next studied the spatial configuration of the above-mentioned genomic segment (Figure 2A) using both the M3C and the standard 3C protocols to directly compare results from these two different methods (Figure 2B and C). The anchor was fixed on the CpG island that contains the promoter for the presumed housekeeping gene, *C16orf35* (29,30).

In the standard 3C experiment (Figure 2B), the anchor DNA fragment preferentially interacted with DNA fragments that contained the α^D gene promoter, the CpG island located upstream of the erythroid cell-specific *TMEM8* gene and the CpG islands associated with the promoters of the housekeeping genes *MPRL28* and *AXIN1* in both HD3 and DT40 cells. Additionally, in proliferating HD3 cells, the anchor fragment demonstrated an increased association frequency with the major regulatory element (MRE) (29) of the α-globin gene domain. The frequency of association with the α^D gene promoter was approximately two times higher in HD3 cells compared to DT40 cells, and it was further augmented in differentiated HD3 cells. The frequency of interaction of the anchor fragment with the fragment bearing MRE was also increased about two times upon differentiation of HD3 cells. These data agree with previously published results (6). A different pattern of associations for the DNA fragments of the chromosomal region being studied was observed in the M3C experiment (Figure 2C). There was a clear interaction between the anchor DNA fragment and the CpG islands of the housekeeping genes *MPRL28* and *AXIN1* in DT40 and HD3 cells both before and after induction of terminal erythroid differentiation of the latter. The interactions between the anchor fragment and the MRE and the α^D gene promoter were no longer present. In DT40 cells, the CpG island located upstream of the *TMEM8* gene, but not the gene itself, was clearly associated with the anchor fragment. This association was also present, but less pronounced, in proliferating and differentiated HD3 cells.

To accurately interpret the results of the M3C experiments, it is necessary to consider the possibility that there is cross-ligation of nmDNA fragments that are not located in close proximity to each other on the nuclear matrix. The diameter of the HD3 cell nucleus/nuclear matrix is ~10 μm, which translates into a 29.4-kb linear DNA fragment. As was mentioned above, we cleaved the gene locus under study into a set of DNA fragments that ranged in size from 0.02 to 7.7 kb by treating the nucleoids with a combination of the restriction enzymes BamHI and BglII (see the scheme in the Figure 2A). The anchor was located on the 5.9-kb fragment. Depending on the position of the attachment site, the end of this fragment could reach an area that is 1–2 μm away from the attachment site (this distance corresponds to 10–20% of the nuclear diameter). In this situation, one cannot exclude the possibility of cross-ligation between DNA fragments that are not assembled into a single chromatin hub (Figure 3, scheme in the top, left side, regions inside dotted circles). However, this will not occur if the nmDNA fragments are reasonably short or if their mobility is restricted by multiple associations with the nuclear matrix (Figure 3, scheme in the top, right side). To determine if any of the apparent associations between the nmDNA fragments that were observed in the M3C experiment above (Figure 2C) can be explained by cross-ligation between DNA fragments that are assembled in different chromatin hubs, the following experiment was performed. The nuclear matrices were isolated following the standard protocol and then fixed with 1% formaldehyde. After fixation, the nuclear matrices were lysed with 1% SDS, which was then sequestered by Triton X-100, and cross-ligation of DNA fragments was performed

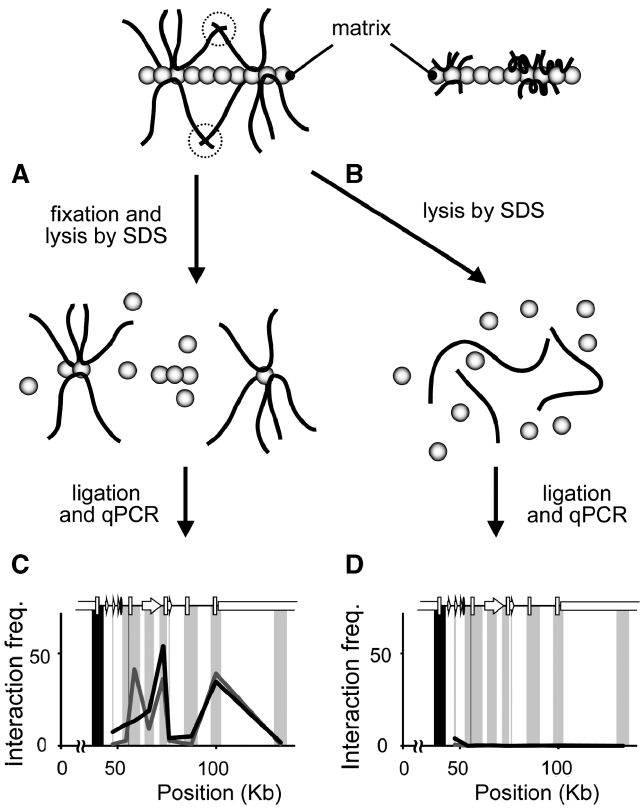

Figure 3. A schematic illustrating the possibility of cross-ligation of nmDNA fragments from different nuclear matrix-bound chromatin hubs, and the experimental approach used to test this possibility. (**A**) A schematic showing the main steps of the control experiment with formaldehyde fixation of the nuclear matrix DNA complexes and subsequent analysis of ligation frequencies by normal 3C protocol. See the text for the details. (**B**) A schematic showing the main steps of the control experiment with lysis of non-fixed nuclear matrix with SDS. (**C** and **D**) Graphs showing the frequencies of interactions of the anchor placed on the *C16orf35* CpG island with the downstream DNA fragments observed in experiments outlined in sections (A) and (B) correspondingly.

followed by dilution according to the standard 3C protocol (Figure 3A). We assumed that the nuclear matrices would disintegrate in the presence of SDS because this occurs with fixed nuclei in the standard 3C procedure. Correspondingly, the remnants of the chromatin hubs (DNA fragments that are linked to each other by nuclear matrix proteins) would be separated, and the possibility of cross-ligation between fragments that are assembled in different chromatin hubs would be negligible after dilution. When the experiment described above was performed and the frequencies of ligation of an anchor fixed within the *C16orf35* CpG island to downstream DNA fragments were determined (Figure 3C), the same profile of associations was observed as in the experiment with direct ligation of nuclear matrix-bound DNA fragments (compare Figures 2C and 3C). Therefore, cross-ligation between nmDNA fragments that are initially assembled in different chromatin hubs and located at a considerable distance from each other did not occur, at least within the area studied in our experiments. Nevertheless, we performed an additional control experiment. In this experiment, we analyzed the possibility of ligation of the anchor placed on the *C16orf35* CpG island with nmDNA fragments from different chromosomes. The fragments harboring the CpG islands for the *c-Myc* (chromosome 2) and *ERCC3* (chromosome 7) genes were chosen as test fragments in this experiment because the results discussed above (Figure 2B, Supplementary Figure S1) and the data presented below (Figure 4) suggest that CpG islands that harbor the promoters of housekeeping genes are bound to the nuclear matrix. The frequency of ligation of both fragments to the *C16orf35* CpG island anchor was very low, although the representation of the DNA fragments bearing both the *c-Myc* and *ERCC3* CpG islands in the nmDNA was higher (*c-Myc*) or comparable (*ERCC3*) with that of the fragments bearing *C16orf35*, *MPRL28* and *AXIN1* CpG islands (Table 1).

We performed an additional control experiment in which the nuclear matrices that were prepared according to the standard M3C procedure were lysed with SDS prior to ligation (Figure 3B). In this case, no preferential ligation of the anchor DNA fragment to any of the downstream fragments was observed (Figure 3D). Therefore, the ability to observe nmDNA fragment associations using the M3C protocol is strongly dependent on the integrity of the nuclear matrix.

The main conclusion from the M3C experiments in which the anchor was fixed on the *C16orf35* CpG island is that the CpG islands of housekeeping genes that are present within the area under study form a stable complex, which retains its integrity following the removal of chromatin (i.e. is attached to the nuclear matrix or is an integral part of the nuclear matrix). To further test this conclusion, the anchor was sequentially moved to the CpG islands of the *MPRL28* and *AXIN1* genes. In these M3C experiments, there was again a clear association between the CpG island that was used as the anchor fragment and the two other CpG islands harboring the promoters of housekeeping genes in both erythroid and lymphoid cells (Figure 4A and B). Therefore, the

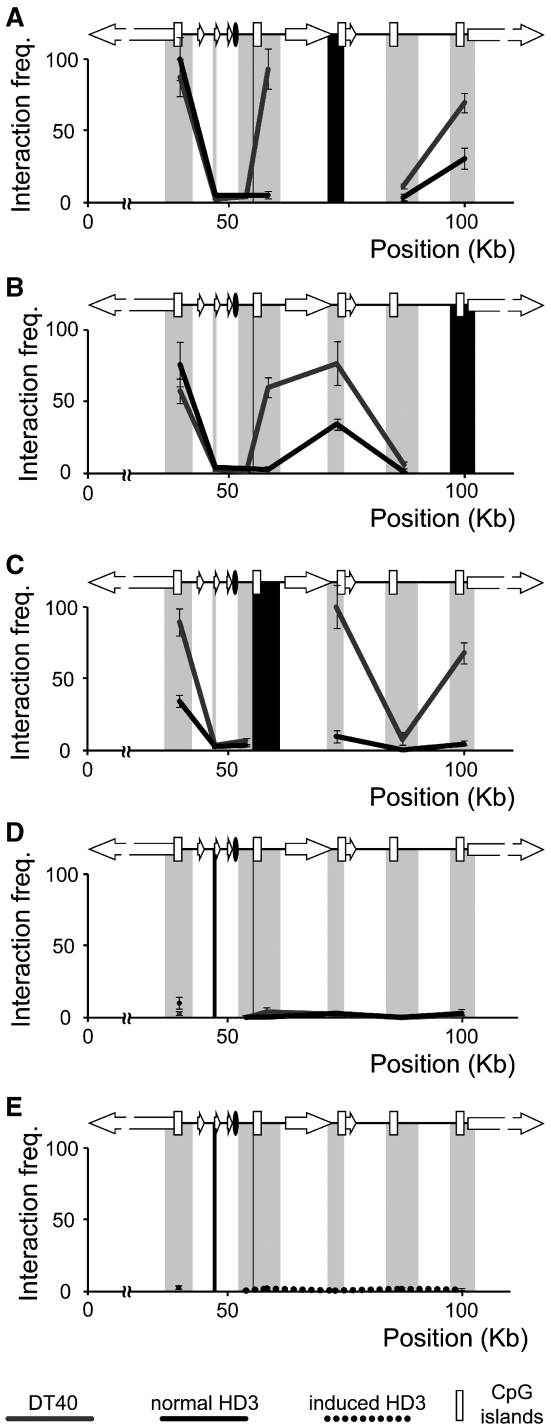

Figure 4. The interaction frequencies observed in the M3C experiments in which the anchor was placed on different DNA fragments. (**A**) Anchor on the CpG island of the *MPRL28* gene, (**B**) Anchor on the CpG island of the *AXIN1* gene, (**C**) Anchor on the CpG island of the *TMEM8* gene, and (**D** and **E**) Anchor on the promoter of the α^D gene. All designations are the same as in Figure 2. Error bars represent the SEM for at least two independent experiments.

CpG islands that are associated with the promoters of housekeeping genes (*C16orf35*, *MPRL28* and *AXIN1*) form a stable complex on the nuclear matrix. The CpG island of the *TMEM8* gene interacted with the CpG

Table 1. M3C analysis of interactions between the *C16orf35* gene CpG island and the CpG islands of housekeeping genes from both the same and different chromosomes

Gene	Chromosome	DT40 cells		Proliferating HD3 cells	
		Content in matrix	Frequency of interaction	Content in matrix	Frequency of interaction
C16orf35 (anchor)	14	20.2		31.6	
MRPL28	14	33.1	100	14.9	100
AXIN1	14	32.8	90	19.4	83
ERCC3	7	26.4	0.7	16.0	1.2
c-Myc	2	100	2.1	100	0.4

The column 'content in matrix' shows the amounts of restriction fragments in the fraction of nmDNA, as determined by TaqMan real-time PCR with primers targeting the internal region of the restriction fragment, normalized to the amount of the *c-Myc* gene CpG island, which was the highest among the five GpG islands tested and was arbitrarily considered to be 100.
The column 'frequency of interaction' shows the interaction frequencies of different CpG islands normalized to the frequency of interaction between the *C16orf35* and *MPRL28* CpG islands, which was the highest frequency observed in all experiments and was arbitrarily considered to be 100.
A random ligation template was prepared by mixing equimolar amounts of PCR-amplified DNA fragments that span the restriction sites of interest, digestion with restriction enzymes and re-ligation.
The sequences of PCR primers and TaqMan probes are listed in the Supplementary Tables S1 and S2.

islands of both the *MPRL28* and *AXIN1* genes on the nuclear matrix in lymphoid, but not erythroid, cells (Figure 4A and B). This observation was confirmed by an M3C experiment in which the anchor was placed in the *TMEM8* gene CpG island (Figure 4C). Interestingly, the CpG island that is located between the *MPRL28* and *AXIN1* genes does not contain a promoter and did not interact on the nuclear matrix with any of the other four CpG islands studied in our experiments.

The M3C experiments in which the anchor was placed within either the *MPRL28* or *AXIN1* CpG islands demonstrated that none of these CpG islands interact with the α^D gene promoter on the nuclear matrix (Figure 4A and B). This observation was further confirmed in experiments in which the anchor was fixed on the α^D gene promoter itself (Figure 4D). These interactions did not change after the terminal erythroid differentiation of HD3 cells was induced, an event that causes significant stimulation of α^D gene expression (Figure 4E).

DISCUSSION

It has become increasingly evident that the eukaryotic genome maintains cell type-specific, functionally dependent spatial organization. This system both ensures and depends on the interaction of distant regulatory DNA sequences (31). Active chromatin hubs are the key elements of this organization, which can be called the 'Functional Architecture' of the eukaryotic genome (4). The organization of multiple regulatory elements that can be located at considerable distances from each other into a single active chromatin hub is mediated by the looping of spacer DNA fragments. The looped DNA fragments can vary in size and can harbor genes and regulatory sequences that do not participate in the assembly of a particular active chromatin hub (5,32). It is not quite clear how the functional architecture of the genome is structurally supported. The simplest possibility is that the interaction between transcription factors that are bound to distant regulatory sequences can bring these sequences together. Alternatively, it was proposed that the functional architecture of the genome is supported by the interaction of special proteins, such as CTCF in vertebrates (33–35) and Su (Hw) in Drosophila (36,37), which are anchored to specific sites in the genome. Interestingly, both proteins have been reported to at least partially reside at the nuclear matrix (38–40). Therefore, it seems logical to ask whether the nuclear matrix as a whole plays a specific role in maintaining the functional architecture of the genome.

As outlined in the 'Introduction' section, the nature of the nuclear matrix is not completely understood. Some scientists even doubt its existence. Recent studies, in particular the characterization of the nuclear matrix proteome (41–43) and detailed ultra-structural studies performed using new experimental approaches (15,44), support the idea that the nuclear matrix (i) exists in living cells and (ii) constitutes a structural milieu for the functional compartmentalization of the eukaryotic cell nucleus (45,46). Earlier studies suggested that the eukaryotic genome is organized into loops that are attached to the nuclear matrix (47). Many researchers have studied the specificity of this organization and have produced conflicting results. Certain DNA sequences were found to possess an ability to interact *in vitro* in a specific fashion with some affinity sites present in isolated nuclear matrices. These DNA sequences were named Matrix Association Regions or MARs (48). The same DNA sequence elements isolated by a different experimental procedure which includes extraction of nuclei with lithium diiodosalicylate (LIS) were named Scaffold Attachment Regions (SARs) (49). In the present literature, the above DNA sequence elements are frequently referred to as S/MARs (50). Although S/MARs are commonly believed to attach DNA loops to the nuclear matrix, this was never proven experimentally. On the contrary, it has been demonstrated that S/MARs are electroelutable from encapsulated and digested nuclei in physiological salt concentration (51), an observation incompatible with a suggestion that S/MARs are involved in DNA loop anchorage to the nuclear matrix. To find out what DNA sequences are attached to the nuclear matrix *in vivo*, we developed a protocol for mapping DNA loop anchorage sites by topoisomerase II-mediated DNA loop excision (52,53). The validity of the proposed protocol was verified by direct visualization of the nuclear matrix-bound DNA loops that were initially mapped by topoisomerase II-mediated DNA loop excision (54). For the present discussion it is important to note that DNA loop anchorage region mapped by topoisomerase II-mediated

DNA loop excision were frequently located in CpG islands, including the CpG island of the chicken *C16orf35* gene (55) and CpG island of the human *c-Myc* gene (53).

In this paper, we attempted to determine if and how the nuclear matrix-bound DNA loops are related to the functionally dependent DNA loops that could be mapped by the 3C procedure. The theory behind the M3C protocol is based on the operational definition of the nuclear matrix (10). If the nuclear matrix and the complexes of DNA that associate with the nuclear matrix are both resistant to nuclear extraction using a 2 M sodium chloride solution, then the ligation frequencies of the nuclear matrix-bound DNA fragments can be analyzed independent of a fixation procedure. The observation that an experimental protocol based on the properties of the nuclear matrix allows for the identification of specific interactions between certain genomic regions strongly corroborates the whole conception of the nuclear matrix.

We have applied the M3C protocol to study the role of the nuclear matrix in the spatial organization of an ~130-kb fragment of chicken chromosome 14, which harbors the α-globin gene domain. As our previous experiments exploring the spatial organization of this genomic region were performed using the standard 3C protocol (6,56), we decided to apply the M3C protocol to the same model system for comparison. The present study demonstrates a strong association of several CpG islands that are present within the genomic locus being studied on the nuclear matrix. These CpG islands are associated with the housekeeping genes *C16orf35*, *MPRL28* and *AXIN1*. There is also an interacting CpG island that is located upstream of the *TMEM8* gene. The latter seems to be released from the complex in erythroid cells. The other three CpG islands are located on the nuclear matrix in close proximity to each other in both erythroid and lymphoid cells. It is likely that the nuclear matrix-bound complex of CpG islands is a transcription factory or part of a transcription factory that might include CpG islands bearing the promoters of additional genes that are located beyond the genomic area studied in our experiments. To this end, it is important to mention that *C16orf35*, *MPRL28*, *AXIN1* and *TMEM8* genes are transcribed in both erythroid and non-erythroid cells (56). On the contrary, an isolated CpG island that is located between the *MPRL28* and the *AXIN1* genes and is not associated with any gene is not attracted to the aforementioned nuclear-matrix bound complex of CpG islands. Thus, the assembly of several CpG islands into a nuclear matrix-bound complex correlates with the transcription of housekeeping genes associated with these CpG islands. We know very little about the organization of transcription factories (57,58). In fact, there is only evidence of clusterization of transcribing RNA polymerase II molecules. However, it has been reported that transcription factories are assembled on the nuclear matrix (21), and our data are in agreement with this observation. Studies based on the spatial organization of the mouse α-globin gene domain suggest that the promoters of globin genes are recruited to the preexisting transcription factory that mediates transcription of the surrounding housekeeping genes upon globin gene activation (9). Our data [Figure 1B, (6,56)] suggest that the same is true for chicken α-globin genes. However, the interactions between globin gene promoters and the CpG islands of flanking housekeeping genes were detectable in erythroid cells by normal 3C analysis but were not detectable in M3C experiments. This observation correlates well with a very low abundance of erythroid-specific elements in nuclear matrix DNA isolated from either erythroid (HD3) or non-erythroid (DT40) cells (see Supplementary Figure S1). Therefore, these interactions are not stabilized by the nuclear matrix and are not resistant to high salt extraction. This is not a specific property of proliferating HD3 cells in which globin genes are transcribed at basal levels and the active chromatin hub controlling the expression of globin genes is not yet fully assembled (6). Upon differentiation, the expression levels of globin genes in HD3 cells dramatically increases, and the promoter of the α^D gene becomes a part of the globin gene active chromatin hub, which includes the CpG island of the *C16orf35* gene and, most likely, the CpG islands of several other housekeeping genes [Figure 2B in this paper, (6,56)]. However, the α^D gene promoter is released from this chromatin hub when the nuclear matrix is isolated from the HD3 cells induced to a terminal erythroid differentiation. In induced HD3 cells, the M3C experiments did not reveal an association between the α^D gene promoter and any of the surrounding CpG islands (Figure 3E). This argues that there are different compartments within a single transcription factory: a basic or permanent compartment composed of promoters of housekeeping genes that are integrated into the nuclear matrix and a 'guest' compartment where promoters of tissue-specific genes can be recruited (see the model in Figure 5). At first sight, the proposed model is in contradiction with the observation that the *TMEM8* gene CpG island is present in the nuclear matrix-bound complex of the housekeeping gene promoters in lymphoid (DT40) cells. Although this gene does not encode a globin chain, it demonstrates an erythroid-specific expression pattern in chickens, which is controlled by some regulatory sequences of the α-globin gene domain (56). However, the fact that the *TMEM8* gene is expressed in various tissues in other vertebrates should not be ignored (59). The *TMEM8* gene might, in fact, be a housekeeping gene. In chickens, *TMEM8* was relocated to the downstream flank of the α-globin gene domain by the inversion of a relatively long genomic fragment and eventually was incorporated into the regulatory network of the α-globin gene domain (56). However, this gene has an upstream CpG island, and it is possible that it still retains some special features necessary for its integration into the nuclear matrix-bound transcription factory. The functional significance of the division of a transcription factory into two compartments is not clear at the moment. It might be that high levels of transcription are incompatible with the integration of a gene into the nuclear matrix. This might explain why the CpG island of the *TMEM8* gene is not present in the nuclear matrix-bound complex of CpG islands in erythroid cells.

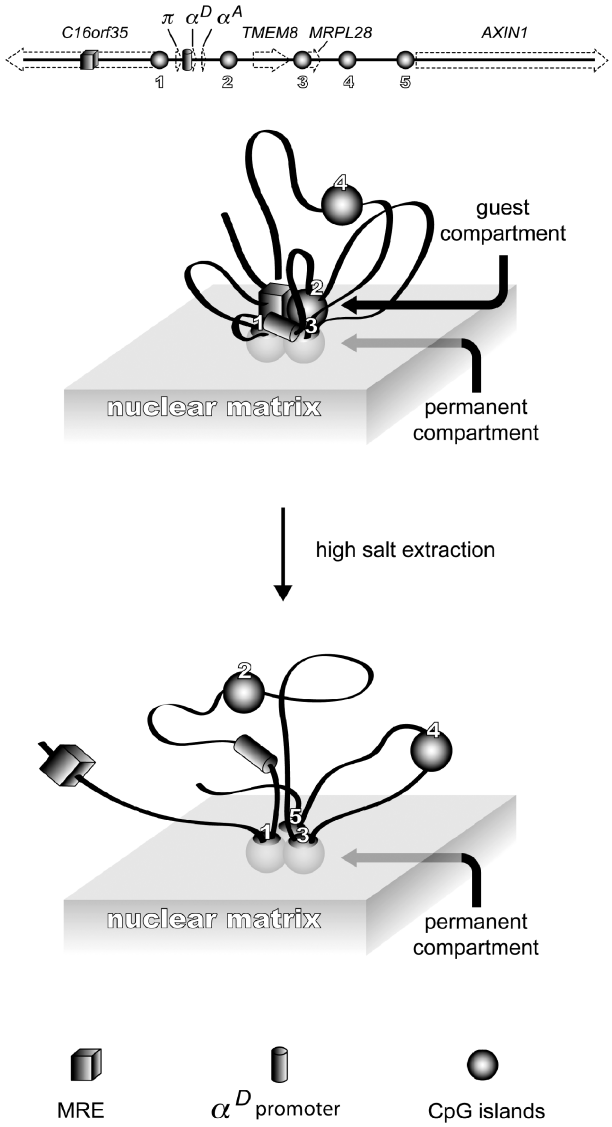

Figure 5. A schematic illustrating the model for a nuclear matrix-bound transcription factory in erythroid cells. CpG islands bearing promoters for the housekeeping genes *C16orf35*, *MPRL28* and *AXIN1* (indicated in the schematic Figures 1, 3 and 5) constitute a permanent compartment of a transcription factory. They are permanently bound to (partially integrated into) the nuclear matrix. The elements of a 'guest compartment' (the α^D gene promoter, the CpG island of the *TMEM8* gene and the erythroid-specific major regulatory element) interact with the surface of the permanent compartment that is not immersed into the nuclear matrix. These interactions are not resistant to 2M NaCl extraction and become disassembled in a result of this extraction.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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