A Functional Role for 4qA/B in the Structural Rearrangement of the 4q35 Region and in the Regulation of FRG1 and ANT1 in Facioscapulohumeral Dystrophy

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Abstract

The number of D4Z4 repeats in the subtelomeric region of chromosome 4q is strongly reduced in patients with Facio-Scapulo-Humeral Dystrophy (FSHD). We performed chromosome conformation capture (3C) analysis to document the interactions taking place among different 4q35 markers. We found that the reduced number of D4Z4 repeats in FSHD myoblasts was associated with a global alteration of the three-dimensional structure of the 4q35 region. Indeed, differently from normal myoblasts, the 4qA/B marker interacted directly with the promoters of the FRG1 and ANT1 genes in FSHD cells. Along with the presence of a newly identified transcriptional enhancer within the 4qA allele, our demonstration of an interaction occurring between chromosomal segments located megabases away on the same chromosome 4q allows to revisit the possible mechanisms leading to FSHD.

Introduction

Facio-scapulo-humeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disease characterized by weakness and atrophy of muscles of the face, upper arms and shoulder girdle. In patients with FSHD, a deletion in a polymorphic locus of the chromosome 4q reduces the number of D4Z4 repeats to less than 10 vs up to 200 in normal individuals [1]. Each 3.3 kbp D4Z4 element harbors DUX4, a gene which encodes a double homeodomain protein [2-4]. Three other genes FRG1 (FSHD Region Gene 1) [5,6], FRG2 (FSHD Region Gene 2) [5,7] and ANT1 (Adenine Nucleotide Translocator 1) [8] are located within the 4q35 chromosomal region and have been reported to be upregulated in FSHD patients. Abrupt expression of FRG1, which is thought to encode a splicing regulator [6,9], could explain the simultaneous changes in expression of many genes. Nevertheless, the evidence of their involvement in FSHD pathogenesis is missing. Some studies even argue against the upregulation of FRG1 and FRG2 in FSHD muscles [10,11]. Indeed, to date, the many proteomics and transcriptome approaches have provided a wealth of data suggesting that the contraction of the D4Z4 repeat array is not sufficient to cause the disease and that FSHD is likely to be a multifactorial disorder (reviewed in [12]).

Several years ago a transcriptional repressor was identified within the D4Z4 repeat array [5]. However, we have recently demonstrated that overall, each D4Z4 repeat has an enhancer activity due to the presence of a very strong enhancer [13]. Moreover, we have shown that a nuclear matrix attachment site (S/MAR), which is positioned in the immediate vicinity of the D4Z4 repeat array [14], may function as an insulator and block the D4Z4 enhancer in normal, but not FSHD, cells [13]. In fact, this S/MAR is prominent in normal myoblasts and non-muscular human cells, and much weaker in muscle cells derived from FSHD patients [14]. From this observation, we inferred that, in normal human myoblasts, the D4Z4 repeat array and neighboring genes are located in two distinct loops, whereas, in myoblasts from FSHD patients, they are in a single one. This suggests that a looping mechanism could lead to a direct contact between the D4Z4 array and genes that are positioned in cis on the chromosome but are too far away to be subjected to transcriptional regulation through classical molecular mechanisms [14].

Intriguingly, FSHD occurs only in individuals bearing the 4qA allele. 4qA/B is a 10 kb-long polymorphic segment directly adjacent to the D4Z4 repeat array. It exists in two allelic forms, 4qA and 4qB, which are 92% identical and equally common in the general population [15,16]. The main difference between the two alleles resides in a tract of β-satellite repeats present in 4qA but not 4qB [15]. This dissimilarity may bear consequences either in the predisposition to deletions occurring within the D4Z4 repeat array or in the structural consequences of the deletion.

Here, we have further investigated the three-dimensional structure of the 4q subtelomeric region using the recently described 3C technique. We now report significant differences existing between FSHD and normal muscle cells.
Role of 4qA/B Marker in FSHD

A

B

C

D

E

Control

FSHD

Primer pair:

Primer pair:
Results

3C analysis of DNA-DNA interactions at 4q35 in normal human myoblasts

The 3C technique evaluates the spatial proximity of two genomic fragments based upon their relative propensity to get crosslinked in vivo [17–19]. The method uses a restriction enzyme to digest previously crosslinked chromatin. After ligation of very dilute DNA to favor intramolecular rather than intermolecular ligation of crosslinked DNA, the ligated fragments are amplified by PCR using specifically designed primers. In the present study, we have used the BglII enzyme whose recognition sequence is present on average every 3,500–1,500 bp within the studied region. Such a DNA length is appropriate for the 3C assay.

We selected several genes and landmarks (Figure 1A) within the 5 Mb-long subtelomeric region of chromosome 4q to study their propensity to get crosslinked in vivo. These included 4qA/B, a distal segment adjacent to the polymorphic 4qA/4qB marker [15,16]; D4Z4, a 3.3 kb fragment containing the D4Z4 repeat array itself; FR-MAR, the fragment containing the S/MAR whose function is weakened in FSHD muscle cells [14]; 5'NT, a non-transcribed fragment located between the D4Z4 array and the FRG1 gene promoter, respectively [20]; and ANT1, the gene promoter.

We next performed the same 3C analysis using myoblasts derived from an FSHD patient. Differently from what observed in normal muscle cells, we could not detect any interaction between FRG1-1 and FRG2 and DUX4c. Specifically, DUX4c strongly interacted with the distal part of the promoter of FRG1 (FRG1-1) and, to a lower extent, with the promoter of FRG2, and also with the subtelomeric region proximal to the 4qA/4qB marker. FR-MAR and 5'NT did not interact with other landmarks, whereas D4Z4 interacted only with the region proximal to DUX4c. Thus, in normal myoblasts, we have found that the D4Z4 repeat array does not directly interact with any gene promoter.

3C analysis of DNA-DNA interactions within 4q35 in FSHD myoblasts

We next performed the same 3C analysis using myoblasts derived from an FSHD patient. Differently from what observed in normal muscle cells, we could not detect any interaction between FRG1-1 and FRG2 or FRG1-2, whereas we consistently identified a novel interaction between FRG1-1 and 4qA/B. Indeed, in FSHD myoblasts, the 4qA/B landmark strongly interacted not only with DUX4c (as in control cells), but also with FRG1-1, FRG1-2 and the promoter of the 4qA/B marker. FR-MAR and 5'NT did not interact with other landmarks, whereas D4Z4 interacted only with the region proximal to DUX4c. Thus, in normal myoblasts, we have found that the D4Z4 repeat array does not directly interact with any gene promoter.

The majority of the interactions detected in the 3C assay occur in cis within 4q35

The data obtained with the 3C assay evidence the spatial proximity of sequences along the subtelomeric region of chromosome 4q. However, approximately 60 kb of sequences within this region are also present on chromosome 10q which
The 4qA allele contains a transcripational enhancer

We then asked whether the 4qA/B marker, which in FSHD myoblasts interacts directly with the promoters of FRG1 and ANT1, could have a role in the transcriptional regulation of these two genes. Since all FSHD patients carry the 4qA phenotype on the deleted 4q chromosome [15], we tested whether the 4qA allele could directly regulate gene transcription. To this aim we cloned the 4qA marker in both orientations in the pGL3-promoter plasmid, a luciferase reporter vector. We transfected constructs and control plasmids in HeLa cells, and then measured reporter gene expression 48 hours after transfection. The presence of the SV40 enhancer in the positive control (pGL3con, Figure 3B) resulted in a five-fold increase of the transcription levels in comparison to the enhancer-less control plasmid (pGL3Pro). The 4qA fragment cloned into the enhancer-less pGL3-promoter plasmid stimulated luciferase synthesis with 60% efficiency as compared to the SV40 enhancer positive control. Thus, the 4qA allele exhibited properties of a transcripational enhancer. This enhancer was also active in a cell line derived from a human rhabdomyosarcoma, a tumor of muscular origin (data not shown).

Discussion

Despite many studies performed in the last twenty years, the mechanism leading to the emergence of FSHD remains poorly understood. The 3C data reported here provide the first experimental evidence that, in this genetic disease, molecular events occur that involve chromosomal segments located at a very large linear distance on the partially deleted chromosome 4q. Specifically, we have observed that in FSHD myoblasts, the subtelomeric 4qA/B marker strongly interacts with the promoter of the FRG1 gene which is located dozens of kbp proximally on the chromosome, depending on the number of remaining D4Z4 repeats. Even more strikingly, we documented a direct interaction of 4qA/B with the promoter of the ANT1 gene which lies at a linear distance greater than 5 Mbp on the centromeric side. This interaction is FSHD-specific as, in control myoblast cells, the 4qA/B marker did not interact with the FRG1, or the ANT1 promoters. 4qA/B is a 10 kb-long polymorphisic segment directly adjacent to the D4Z4 repeat array. It exists in two allelic forms, 4qA and 4qB, which are 92% identical and equally common in the general population. FSHD, however, has been reported to occur only in individuals with the 4qA allele [15,16]. The main difference between the two alleles resides in a tract of β-satellite repeats present

Table 1. Frequencies of cis-interactions within 4q35 in normal and FSHD myoblasts.

| Primer | ANT1 | FRG1-1 | FRG1-2 | DUX4c | FRG2 | B'NT | FR-MAR | D4Z4 | 4qa/b |
|--------|------|--------|--------|-------|------|------|--------|------|-------|
|        | 1    | +      |        |       |      |      |        |      |       |
| FRG1-1 | 2    | +      | -      | +     | -    | +    |        |      |       |
| FRG1-2 | 3    | -      | +      | +     | +    | +    |        |      |       |
| DUX4c  | 4    | +      | +      | +     | +    | +    |        |      |       |
| FRG2   | 5    | -      | +      | +     | +    | +    |        |      |       |
| B'NT   | 6    |        |        |       |      |      |        |      |       |
| FR-MAR | 7    |        |        |       |      |      |        |      |       |
| D4Z4   | 8    | +      |        |       |      |      |        |      |       |
| 4qa/b  | 9    | +      |        |       |      |      |        |      |       |

Horizontal and vertical dashes indicate interactions detected by the 3C technique in normal and FSHD myoblasts, respectively.

Normal Myoblasts.
FSH Myoblasts.
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FRG2 promoter regions in both normal and FSHD myoblasts regulate the transcription of the D4Z4 enhancer, within the D4Z4 repeat array, may directly within the D4Z4 repeats [2,3,25]. Thus, our results suggest that the and DUX4c are two genes that have been shown to be transcribed promoters of these three genes [5,7,13,14] appears unlikely. DUX4 regulation through a direct contact of the D4Z4 array with the FRG1 and FRG2 genes. In accordance, the hypothesis of a transcriptional enhancer. Moreover, DUX4 interacts with DUX4c which, in turn, makes contact with FRG1 and FRG2. This may provide a molecular basis for the transcriptional regulation of neighbor genes by DUX4/DUX4c.

We then detected a new enhancer element in the 4qA allele that may regulate the expression of the FRG1 and ANT1 genes specifically in FSHD cells through a direct interaction with the respective gene promoters. Indeed, both ANT1 and FRG1 are activated in FSHD patients [5,6,8]. It is noteworthy that the (1.5 to 5 fold) up-regulation of these two genes seen in FSHD patients is consistent with the relatively weak effect of the 4qA enhancer in the luciferase assay.

Recently, we have reported that in FSHD myoblasts, the nuclear matrix attachment site FR-MAR was specifically delocalized from the nuclear matrix [14]. In normal cells, this S/MAR may constrain the flexibility of the region by anchoring it to the nuclear matrix, thus restricting interactions of adjacent sequences in the three dimensional nuclear space. This could particularly affect the 4qA/B marker which is separated from neighbor genes by the S/MAR. In FSHD cells, the delocalization of FR-MAR would thus result in an increased flexibility of the corresponding chromosomal segment and additional possibilities of interaction for the 4qA/B marker. This may provide an explanation for the FSHD-specific, direct interaction of 4qA/B with the ANT1 and FRG1 gene promoters we observed in FSHD myoblasts. In the present 3C experiments, no interactions were detected that involved FR-MAR. This should not be surprising since previous 3C studies have already stressed that S/MARs appear to interact only with other SMARs [26].

The experimental approach used here provides new ways to systematically explore the higher-order chromatin structure of any chromosomal region. In this study, we have found that the binding of DUX4c to the FRG1 and FRG2 gene promoters appears to play a key role in structuring the 4q35 region in normal cells. Other interactions take place in FSHD cells and this is the likely result of a global reorganization of the locus in relation with the contraction of the number of D4Z4 tandem repeats. This reorganization is schematized in the three dimensional model shown in Figure 4. In FSHD cells (Figure 4B), the deletion of D4Z4 repeats and the delocalization of the proximal S/MAR would result in the formation of a giant loop where the subtelomeric 4qA/B sequence is now brought in close proximity not only to DUX4c and FRG1 but also to the proximal ANT1 gene promoter which lies 5 Mb away on the centromeric side of the region. This major structural rearrangement, as compared to the normal situation (Figure 4A), would make gene promoters accessible to the DUX4c and 4qA enhancers specifically in FSHD myoblasts. One hypothesis to explain how such long-range changes in higher order chromatin structure can occur relates to differences in the methylation status of the corresponding regions [27,28]. Further studies are clearly needed to explore this and other hypotheses.

Materials and Methods

Cell lines

The HeLa cell line was purchased from the ATCC collection. The GM10115A hybrid murine cell line containing the human chromosome 4 was a kind gift of Dr. Rosella Tupler. Primary muscle fibroblasts from two different healthy individuals and two FSHD patients with 5.5 D4Z4 repeats and 7 repeats [14,29] in the

in 4qA but not 4qB [15]. This difference may bear consequences either in the predisposition to deletions occurring within the D4Z4 repeat array or in the pathological consequences thereof.

Another surprising observation was that, in both normal and FSHD cells, the D4Z4 marker interacted only with its related sequence DUX4c among the various segments tested. No interactions were detected with the promoter regions of ANTI1, FRG1 or FRG2. In accordance, the hypothesis of a transcriptional regulation through a direct contact of the D4Z4 array with the promoters of these three genes [5,7,13,14] appears unlikely. DUX4 and DUX4c are two genes that have been shown to be transcribed within the D4Z4 repeats [2,3,25]. Thus, our results suggest that the D4Z1 enhancer, within the D4Z4 repeat array, may directly regulate the transcription of the DUX4 and DUX4c genes.

We then found that DUX4c crosslinked with the FRG1 and FRG2 promoter regions in both normal and FSHD myoblasts (Figure 2). We therefore postulate that DUX4c plays a key role in the three-dimensional organization of the locus. Sequence alignment analysis (data not shown) suggests that DUX4c contains a transcriptional enhancer. Moreover, DUX4 interacts with DUX4c which, in turn, makes contact with FRG1 and FRG2. This may provide a molecular basis for the transcriptional regulation of neighbor genes by DUX4/DUX4c.
4q35 array, respectively, were cultured on a collagen-coated support in DMEM supplemented with 20% bovine fetal serum.

3C assay
The 3C assay was performed as described elsewhere [30] with some specific adaptations for myoblast cells. Nuclei were prepared using 2 volumes of ice-cold MES lysis buffer [31] for 1 volume of packed cells; a protease inhibitors cocktail (Roche, Complete Mini) was added immediately prior to use. The lysis of nuclei was checked under a microscope. Formaldehyde (Sigma) was added to diluted nuclei (final concentration of $1 \times 10^{-7}$ /ml) to perform the crosslinking. Nuclei were then diluted tenfold and digested overnight at

Figure 4. A–B. Models schematizing the proximity between 4q subtelomeric fragments in control (A) and FSHD (B) nuclei.
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37°C with BglII (New England BioLabs). The BglII restriction sites occur with an average frequency 3500 bp±1500 bp within the 4q35 locus, which is appropriate for the 3C assay.

Digestion mix was inactivated by adding SDS and digested DNA ligated overnight at a low concentration with T4 DNA ligase (Fermentas). Ligation products were detected by PCR amplification using fragment-specific primers. PCR products were separated on 2% agarose gels; images acquired using a Bio-DOC apparatus (Vilbourn-Lourmat, France) and quantified using the Image Gauge 4.0 software (Fuji, Japan).

Three independent controls were carried out using genomic DNA from FSHD myoblasts, normal myoblasts and from the murine hybrid cell line containing the human chromosome 4 as the only human material. The DNA fragments spanning the BglII restriction sites were mixed in equimolar amounts as described elsewhere [18] and added to the appropriate non-crosslinked genomic DNA.

Relative crosslinking frequencies for combinatorial interactions were calculated as the ratio of the amount of product detected with crosslinked DNA template to the amount of product obtained with non-crosslinked, control DNA templates [17,30]. The experiments were carried out in triplicate and were averaged. Data from two independent experiments are presented.

3C primer design

The primers spanning the BglII sites were designed using OLGIGO Primer Analysis Software 6.71 at positions shown in Figure 1A. Primer sequences are shown in Table S1.

FISH analysis

The p13E11 probe was derived from the pGEM42 plasmid [22] and labeled with biotin-14-4CTP. Hybridization on slides was performed as described earlier [32] using anti-biotin mouse antibodies conjugated with AlexaFluor 488 (Invitrogen, USA). Nuclei were counterstained with 0.5 μg/ml 4,6-diamido-2-phenylindole (DAPI) and mounted using Vectashield antifade mounting medium (Vector Laboratories, USA). Slides were examined under an Olimpus Provis fluorescence microscope with a 100× oil immersion objective and the appropriate filters. Images were captured with a CCD camera (Photometrics, USA), using the RSImage software (Scanalytics, USA).

Vectors and cloning

A series of pGL3 vectors (Promega, USA) was used for transient transfection studies. The pGL3-Promoter vector contains an SV40 promoter upstream of the luciferase gene. The pGEM42 plasmid containing the fragment of chromosome 4 corresponding to the allelic variant 4qA [22] (a kind gift of Dr. A. Belaev) was digested by BamHI and EcoRI (Fermentas, Lithuania). The 598 bp fragment was blunt-ended by Klenow (Fermentas, Lithuania) and cloned in two orientations upstream of the promoter region of the reporter plasmid pGL3-Pro (Promega, USA) digested by SmaI resulting in the plasmids pGL3-4qA1 and pGL3-4qA2.

The pGL3-Control vector contains the SV40 promoter and enhancer sequences, resulting in strong expression of the reporter gene in many types of mammalian cells. Therefore, it was used as a positive control in the experiments on the identification of a putative enhancer within the D4Z4.

Luciferase assay

HeLa cells were plated in 24 well/plates 24 hours before transfection at the density of 50,000 cells per well. The plasmids used for transfection were purified with the Nucleobond midiprep kit (Machery Nagel, Germany) and 1 μg of each was transfected using JetPEI (Polyplus Transfections Inc., USA). 48 hours after transfection luciferase activity was measured with the Luciferase Assay System (Promega, USA) using a Microlumart LB96P luminometer. The protein content of cell extracts was determined with the Quantipro BCA assay kit (Sigma, USA). Each transfection was repeated at least 3 times.

Supporting Information

Table S1 Primers used for the 3C assay.
Table S2 Frequency of pairing between the 4q and 10q in nuclei of normal and FSHD myoblasts.

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Author Contributions

Conceived and designed the experiments: IVP AVP PD. Analyzed the data: IVP AVP PD ML YSV. Contributed reagents/materials/analysis tools: DL. Wrote the paper: IVP AVP PD YSV.

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