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The 4q subtelomere harboring the FSHD locus is specifically anchored with peripheral heterochromatin unlike most human telomeres

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This paper investigates the nuclear localization of human telomeres and, specifically, the 4q35 subtelomere mutated in facioscapulohumeral dystrophy (FSHD). FSHD is a common muscular dystrophy that has been linked to contraction of D4Z4 tandem repeats, widely postulated to affect distant gene expression. Most human telomeres, such as 17q and 17p, avoid the nuclear periphery to reside within the internal, euchromatic compartment. In contrast, 4q35 localizes at the peripheral heterochromatin with 4p more internal, generating a reproducible chromosome orientation that we relate to gene expression profiles. Studies of hybrid and translocation cell lines indicate this localization is inherent to the distal tip of 4q. Investigation of heterozygous FSHD myoblasts demonstrated no significant displacement of the mutant allele from the nuclear periphery. However, consistent association of the pathogenic D4Z4 locus with the heterochromatic compartment supports a potential role in regulating the heterochromatic state and makes a telomere positioning effect more likely. Furthermore, D4Z4 repeats on other chromosomes also frequently organize with the heterochromatic compartment at the nuclear or nucleolar periphery, demonstrating a commonality among chromosomes harboring this subtelomere repeat family.

Introduction

The eukaryotic genome is packaged into large-scale chromatin structures that occupy different subregions of the nucleus associated with either gene activity or gene repression (for reviews see Cockell and Gasser, 1999; Carmo-Fonseca, 2002; Fisher and Merkenschlager, 2002; Parada and Misteli, 2002; Isogai and Tjian, 2003). Euchromatin occupies the internal nucleoplasm, which is further partitioned into several nonchromatin compartments enriched in different nuclear factors with which specific active genes may associate (Shopland et al., 2003). In contrast, heterochromatin forms a compartment of compact DNA at the nuclear periphery, where many inactive genes have been localized not only in yeast (Cockell and Gasser, 1999) but also mammalian (Kosak et al., 2002) nuclei. In human fibroblasts, several inactive loci are found at the nuclear or nucleolar periphery, whereas specific active loci reside internally with SC35 domains (speckles) rich in RNA metabolic factors (Xing et al., 1995), and this organization changes with cell-type gene expression (Moen et al., 2004). Some inactive loci (facultative heterochromatin) associate with constitutive centromeric heterochromatin, which can also be at the nuclear periphery (Brown et al., 1997; Francastel et al., 1999). Studies in yeast indicate that localization at the nuclear periphery contributes to gene repression (Andrulis et al., 1998; Feuerbach et al., 2002; Hediger et al., 2002). Yeast telomeres cluster in the nuclear periphery, which is rich in proteins linked to repression, thus forming a silencing-conducive environment important for the action of silencer elements in telomere position effects (TPEs; de Bruin et al., 2000; Fourel et al., 2002).

Work presented here addresses the organization of human telomeres relative to nuclear compartments and entertains current hypotheses on the mechanism of facioscapulohumeral dystrophy (FSHD). FSHD is an autosomal-dominant myopathy and the third most commonly inherited form of muscular dystrophy (FSH Consortium, 1998; Kissel, 1999). FSHD has been linked to contraction of D4Z4 subtelomeric tandem repeats on chromosome 4q35 below a threshold number of ten (van Deutekom et al., 1993). Although it has been suggested that the 3.3-kb D4Z4 repeat contains an open reading frame for a putative homeobox protein (Gabriels et al., 1999), expression of this has not been established; thus D4Z4 is widely postulated to have a noncoding regulatory function (Bickmore and van der Maarel, 2002).

The online version of this article includes supplemental material.
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Abbreviations used in this paper: FSHD, facioscapulohumeral dystrophy; TPE, telomere position effect.

Supplemental Material can be found at: http://www.jcb.org/cgi/content/full/jcb.200403128/DC1
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Results

Human telomeres generally avoid the nuclear periphery, a region rich in heterochromatin

In a preliminary analysis of the FSHD locus organization in lymphocytes, we noted a high percentage of 4q35 signals (45–50%) at the nuclear periphery; in contrast, a U2 snRNA locus probe showed <10% peripheral. Because the random rotation of lymphocytes in suspension complicates analysis and minimizes the apparent frequency of peripheral signals, we focused our work on primary human cells with a defined dorsal/ventral axis in culture. First, heterochromatic and euchromatic nuclear compartments were defined for human fibroblasts, myoblasts, and differentiated muscle. As shown in Fig. 1, late replicating heterochromatic DNA is concentrated around the nuclear periphery, whereas splicing factor (SC35) domains, associated with many active genomic regions (Shopland et al., 2003), are confined to the more internal euchromatic compartment (Fig. 1 A; Carter et al., 1993). These two compartments are also marked differentially by hnRNA, recently shown to distinguish the inactive from the active X chromosomes (Hall et al., 2002; see Materials and methods). The peripheral rim of the nucleus is consistently devoid of hnRNA in both fibroblasts (Fig. 1 B) and muscle (Fig. 1 C). A second region of heterochromatin abuts the nucleolus, consistent with ultrastructural observations (Comings, 1980) and corroborated by the localization of the heterochromatic inactive X to either the nuclear or the nucleolar periphery (Fig. 1, D and E).

Figure 1. Heterochromatin forms a compartment at the nuclear and nucleolar periphery in human fibroblasts and muscle cells. (A) Late-replicating DNA (labeled by BrdU; green) concentrates at the nuclear periphery, whereas SC35 domains rich in RNA metabolic factors (red) punctuate the nuclear interior. (B) Heterochromatin, primarily at the nuclear periphery and nucleolus, is delineated as the DAPI region (blue) devoid of hnRNA (red). Inset shows magnification of the periphery. The image was deconvolved to minimize out-of-focus light. (C) This myotube highlights the prominent peripheral heterochromatin compartment in five nuclei as described in B. (D) The inactive X chromosome marked by the accumulation of Xist RNA (green) is localized either at the nucleolus or at the nuclear periphery (E).
Thus, in our analysis we considered both the nuclear periphery adjacent to the lamina and the region abutting the nucleolus as common components of the heterochromatic compartment.

To address whether the general distribution of human telomeres in primary myoblasts, muscle, and fibroblasts resembles the clustered peripheral distribution of yeast telomeres or is more internal as in transformed HeLa cells (Luderus et al., 1996), a PNA oligo probe to the TTAGGG repeat was hybridized (Fig. 2 A). Results show the majority (83%) of human telomeres to be in the nucleoplasm, clearly distant from the heterochromatic region encircling the nuclear periphery, as viewed in two dimensions. Although only 17% of telomeres were within 0.6 μm of the nuclear periphery, another 22% position at the nucleolus (largely accounted for by the 10 acrocentric chromosomes carrying rDNA genes). Optical sectioning of cultured muscle confirmed that the vast majority of telomeres are internal and do not abut the nuclear envelope in the X-Y or Z planes (Fig. 2 B). Interestingly, a number of telomeres are tightly juxtaposed to the splicing factor–rich SC35 domains. The ∼60–80 separate telomere signals observed suggests that telomere clustering was limited unlike in yeast, which is consistent with findings in HeLa cells (Nagele et al., 2001; Molenaar et al., 2003).

The 4q35 subtelomere specifically localizes to the heterochromatic compartment, primarily the peripheral nuclear rim

Will the organization of the 4q telomere reflect the broad, largely internal distribution of telomeres or might there be chromosome-specific telomere positioning?

The distribution of the RP11-279 marker specific for 4q35.1 (the distal tip of chromosome 4q) was first examined in diploid human fibroblasts. Signals were deemed peripheral if within ∼0.6 μm from the nuclear edge as seen by DAPI and confirmed by nucleopore or lamin staining (see online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200403128/DC1). As shown in Fig. 3 A, the 4q35 signal is located at the outer edge of the nuclear periphery and occasionally with the fibrillarin-stained nucleolus, where the D4Z4-bearing acrocentric chromosomes cluster (see Relationship of D4Z4 repeats on other chromosomes to the heterochromatic compartment). In fibroblasts, 73% of 4q35 signals associate with the heterochromatic compartment (65% nuclear and 8% nucleolar periphery). Given that FSHD is a muscular dystrophy, cultured human skeletal myoblasts and differentiated myotubes were examined, using procedures optimized for analysis of muscle nuclei (Smith et al., 1999). As shown in Fig. 3 (B and I), 87% of myoblast and 92% of myotube signals contacted either the nuclear or nucleolar periphery, with the vast majority (85% in muscle) at the nuclear periphery.

Interestingly, the 4q35 signals consistently reside at the lateral nuclear edge rather than at the dorsal or ventral nuclear surface, rendering localization at or very near the nuclear envelope apparent by two-dimensional analysis of monolayer and confirmed by three-dimensional analysis (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200403128/DC1). The peripheral compartment around the lateral edge of the nucleus was measured in 10 cells and found to comprise 10.1% of the nuclear volume (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200403128/DC1). This defined organization was not recognized in a previous work in lymphocytes (Nikokur et al., 1996), likely because free rotation of suspension cells makes the peripheral locus appear more variably localized. In our analyses of four cell types, including lymphocytes, peripheral localization was detected in many cell lines examined. The trend was most pronounced in muscle.

The demonstration that the FSHD locus is indeed spatially associated with the heterochromatic nuclear compartment indicates that the function of D4Z4 repeats occurs in a nuclear environment typically associated with gene repression. This finding substantially increases the likelihood that this tandem repeat element, deleted in FSHD, is involved in regulation of the heterochromatic state for genes in the region. Moreover, the aforementioned results demonstrate that specific human telomeres are uniquely organized, with the 4q telomere showing an atypical association with the nuclear periphery (Fig. 3 I), which may also have implications for chromosome-specific human TPEs (see Discussion).

A reproducible orientation of 4q versus 4p in the three-dimensional nucleus: correlation with gene expression but not gene density

Is the 4q35 localization specific to this region of the chromosome, or might it also be seen for the 4p telomere and centromere? The 4q35 marker was strongly more peripheral than either the 4p telomere (Fig. 3 C) or the centromere (Fig. 3 D; 78% vs. 9% vs. 28%, respectively). The centromere commonly (48%) abutted the nucleolus (Fig. 3, D and I), and neither the 4q nor 4p telomeres associated with the centromere. The low frequency of peripheral 4p signals demonstrates that chromosome 4 orients with the long arm pointing to the nuclear enve-
lope, whereas centromere and short arm are more internal. In contrast to the predictable arrangement of larger chromosomal segments (4q, 4p, and 4cen), ordering between three loci within the 4q35 subregion was more variable (Fig. 3, E and F), which is consistent with a random walk model (Yokota et al., 1995). Thus, there is a reproducible orientation to this large (~150-Mb) chromosome arm, even though the path of chromatin folding on a smaller scale is more flexible.

The organizational specificity is further demonstrated by comparison to another individual chromosome, using probes available for 17p and q telomeres. As shown in Fig. 3 G, 85–88% of these telomeres were internal in both fibroblasts and muscle. No consistent orientation of 17p and q arms was noted, although the 17q telomere showed a more consistent (88%) association with the edge of SC35 domains (Fig. 3 H), rich in RNA metabolic factors and associated with high gene activity (Xing et al., 1995; Smith et al., 1999).

Fig. 4 A summarizes our findings for the organization of chromosome 4 and contrasts it with chromosome 17. Results demonstrate that a fundamental aspect of chromosome organization in human cells can involve a defined three-dimensional chromosome orientation for a specific chromosome. This organization shows an interesting relationship with recent data on the profiles of gene density and expression over the chromosome (Fig. 4 B; Versteeg et al., 2003). As considered further in the Discussion, the orientation of chromosome 4 is not simply mirrored by differences in gene density on 4q versus 4p, but shows an intriguing correlation with gene expression.

Distinguishing the normal and mutant alleles in individual nuclei of heterozygous FSHD patient cells

It would be valuable to discriminate the mutant from the normal 4q35 locus in single cells because this distinction was not
explored in earlier localization studies (Winokur et al., 1996; Stout et al., 1999). The intensity of FISH signal is proportional to the DNA target size, and direct comparison of normal and mutant alleles in the same cell minimizes technical differences (Johnson et al., 2000). Therefore, we tested a strategy of hybridizing a 4q35 marker with the D4Z4 probe in two different colors to discern the normal from the mutant FSHD alleles in single cells. In the heterozygous SKFSHD5 lymphoblastoid cell line first tested, the mutant allele carries one D4Z4 repeat, whereas the other allele was within normal range. Both alleles showed a strong 4q35 marker signal, whereas only one associated with a normal bright D4Z4 signal, as seen in both interphase nuclei and metaphase spreads (Fig. 5 A). As shown in the following section, we were able to discern this for other FSHD patient cells that retained more than one copy of D4Z4 (see online supplemental material).

Although not our primary purpose here, we suggest that this molecular cytogenetic assay may prove useful in diagnostic assays for FSHD, which are complicated by D4Z4 and 4q homologous sequences on other chromosomes (Kissel, 1999).

**Analysis of 4q35 localization in primary muscle cultures from FSHD patients**

It was important to investigate muscle cells derived from patients who carry a heterozygous D4Z4 mutation. This was made possible by the generous contribution of muscle biopsy samples from FSHD patients and M. Ehrlich to the Coriell Institute. To facilitate their characterization, we worked in conjunction with the Coriell Institute to characterize the myogenic competence of three primary lines of heterozygous...
FSHD patient cells and one non4q FSHD line (see online supplemental material).

As discussed earlier in this paper, the 4q35 locus was extremely (87–92%) heterochromatic in normal myoblasts or muscle. In heterozygous FSHD patient cell lines, the frequency of association of the mutant allele with the heterochromatic compartment remained high and was not substantially different from the wild-type allele in any cell line examined. Many mutant signals showed the most extreme peripheral position (e.g., Fig. 5 C, arrowhead), and thus did not appear to have moved significantly inward. This finding was further examined in two ways. First, the peripheral heterochromatic rim was delineated by hybridization to hnRNA in FSHD myoblast nuclei in which the 4q35 locus was simultaneously labeled (Fig. 5 B). This procedure confirmed that in heterozygous cells, both 4q35 alleles could be seen within the peripheral rim depleted of hnRNA signal. Second, measurement of the distance from the 4q35 locus to the nuclear envelope revealed no significant difference between the localization of the peripheral alleles whether they be mutant (0.4 ± 0.1 μm) or wild type (0.4 ± 0.2 μm).

Although the overwhelming majority of mutant alleles positioned with peripheral heterochromatin, each of three mutant myoblast lines did show a slight decrease in the peripheral placement of the mutant allele compared with the normal allele, with 4–7% more mutant alleles in the euchromatic compartment. However, these differences were not statistically significant and were not seen in differentiated myotube nuclei where both alleles were highly peripheral (Fig. 5 D). Similarly, for nuclei in which one allele was internal and the other peripheral, the peripheral allele was not consistently the wild-type allele. Collectively, our results suggest that differences between alleles likely reflect normal biological variation.

Certain types of muscular dystrophy have recently been shown to result from a defect in the nuclear lamina (Ostlund and Worman, 2003), fueling speculation over the organization of heterochromatin at the nuclear rim. Thus, we considered the possibility that in FSHD myoblasts there could be a greater tendency for both normal and mutant 4q35 alleles to detach from the peripheral rim. Importantly, we also examined this for a myoblast line from a patient with an alternate form of “non-4q” FSHD (line GM17725) that does not carry the 4q35 D4Z4 contraction (van Overveld et al., 2003). As shown in Fig. 5 (G–I), the frequency of nonperipheral alleles in normal and mutant myoblasts was essentially equivalent. The non4q FSHD line showed some diminution of peripheral signals; however, this may reflect decreased proliferative capacity of these cells (see Discussion and online supplemental material). Although not our primary focus here, we note that staining for nuclear lamin A (not depicted) and nucleoporins did not reveal any obvious defect in the nuclear envelope or lamina in the FSHD muscle cell lines (Fig. 5, H and I).
Peripheral localization is an inherent property of 4q35 and is resistant to perturbation

We explored a fundamental question concerning the nature of the 4q35 localization: a full complement of D4Z4 repeats is not required for peripheral positioning, but is this positioning an inherent property of this telomere? Is this chromosomal region specifically “tethered” or confined indirectly by other constraints of nuclear organization? We investigated this in two ways. First, we determined whether or not 4q35 was similarly organized in a mouse–human somatic cell hybrid line, carrying a full complement of mouse chromosomes but only human chromosomes. As shown in Fig. 6 (A and B), even in this foreign environment, 4q35 remains predominantly peripheral (68–73%) and moderately nucleolar (19%); thus, close to ~90% were with the heterochromatic compartment. Interestingly, 4q35 shows no preferential association with the chromatocenters (Fig. 6 B), easily detectable in mouse due to the affinity of their AT-rich centromeres for DAPI (Fig. 6 A). These results indicate that information within 4q alone is likely sufficient for nuclear partitioning of 4q35.

This finding was further tested using a female t(X;4) human lymphocyte translocation cell line, in which a balanced translocation transfers the tip of 4q35 (4q35.2 to the telomere) onto the p arm of the active X chromosome (Fig. 6 D). Hybridization to metaphase chromosomes verified that two probes (RP11-279K24 and a commercial microsatellite probe) bracket the 4q breakpoint and one Xp21.1 probe spans the Xp21 breakpoint, which is consistent with prior characterization of this translocation (Bodrug et al., 1990). In female control cells, the inactive X was distinguished from the active X by the presence of Xist RNA (Clemson et al., 1996) whereas in X-autosome balanced translocations, the intact X is selectively inactivated (Mattei et al., 1982).

As summarized in Fig. 6 C and shown in E–I, swapping the tip of 4q35 for the tip of Xp modifies the nuclear location of loci on each derivative chromosome. Not only can this piece of 4q35 maintain its peripheral organization, it may also increase the propensity of an adjacent locus to situate at the nuclear periphery. Although the active Xp21 locus is itself somewhat peripheral, the Xp21 locus on the translocated active X (carrying 4q35) becomes distinctly more peripheral (49%) compared with the same locus on the intact active X in normal cells (30%). This substantial increase (66%) was evident despite the random rotation of lymphocytes, which underestimates the number of peripheral loci (discussed in Fig. 6). The Xp21 locus on the translocated active X chromosome is as peripheral (slightly more) as on the inactive X, known to be in peripheral heterochromatin. Similarly, swapping 4q35.2 for a piece of X on the derivative chromosome 4 resulted in a less peripheral distribution (37% vs. 56% for normal chromosome 4, a decrease of over 50%). These results indicate that a strong affinity for the nuclear periphery is an inherent property of the distal ~4 Mb of 4q35 and potentially the 4q subtelomere itself.

Relationship of D4Z4 repeats on other chromosomes to the heterochromatic compartment

The aforementioned results demonstrate that a full complement of D4Z4 repeats is not required for the nuclear localization of 4q35 to the heterochromatic compartment. However, if D4Z4 repeats have some functional relationship to heterochromatin and/or nuclear compartmentalization, other chromosomal loci bearing these repeats may show commonalities in their interphase organization with the nuclear or nucleolar periphery. As depicted in Fig. 7 A, D4Z4 repeats are detected in the subtelomeres of the five acrocentric chromosomes as well as 4q, 10q, and 1q12 (Lyle et al., 1995; Winokur et al., 1996), indicating that D4Z4 comprises a specific family of subtelomeric repeats with unknown function.

Analysis of D4Z4 repeats in fibroblasts (Fig. 7 B) and myoblasts (Fig. 7 C) showed strong commonalities in their interphase organization. Though not invariant, the D4Z4 repeats are found with the nucleolus (61%) or the nuclear periphery (21%) in myoblasts. All five pairs of human acrocentric chromosomes carry D4Z4 repeats very closely linked to rDNA genes, which would be predicted to localize to the nucleolus (Fig. 7 D, arrow). However, even if the nucleolar signals are discounted, D4Z4-contain-
ing loci are still four to five times more likely to occupy a peripheral heterochromatic location than are telomeres in general. This distinction is most directly tested by examination of chromosome 10q, which bears neither rDNA repeats nor a huge block of constitutive heterochromatin (as at chromosome 1q12). As shown in Fig. 7 (C and E), 10q localizes to the heterochromatic compartment in myoblasts (82%), either at the nuclear periphery (64%) or the nucleolus (18%). We have found that in certain cell types specific acrocentric chromosomes do not participate in formation of the nucleolus, but instead localize to the extreme nuclear periphery (unpublished data), which is consistent with the idea that these represent alternate but related regions.

Thus, although in general telomeres avoid the nuclear or nucleolar periphery, loci bearing the D4Z4 subtelomeric repeat family exhibit a common affinity for these regions and constitute a distinct organizational subset of telomeres. Fig. 7 A also demonstrates another common feature of their organization: they reside adjacent to blocks of satellite DNA (constitutive heterochromatin) even when at an interstitial site on chromosome 1, as also apparent in the nucleus of Fig. 5 A. The implications of this organization will be further considered in the Discussion.

Discussion

This paper contributes several new findings regarding basic principles of chromosome organization while also providing insight into potential mechanisms operative in FSHD. The 4q35 locus positions in the heterochromatin-rich compartment in several cell types studied; importantly, this localization is specific to the 4q telomeric region. These results establish that, unlike in yeast, specific human telomeres occupy functionally distinct nuclear domains (e.g., 17q is not only internal, but with SC35 domains rich in RNA metabolic factors). Thus, distinct gene regulatory phenomena may be linked to specific human telomeres. TPEs are well established in yeast telomeres, which more uniformly cluster at the nuclear periphery where repressive chromatin factors accumulate. Studies indicate this organization is important in at least one type of TPE (Andrulis et al., 1998; Feuerbach et al., 2002; Hediger and Gasser, 2002). Our results are consistent with the finding that human telomeres are generally not late replicating (Wright et al., 1999), leading to speculation that TPEs may apply only to specific telomeres. Although TPE has been more difficult to demonstrate in human cells, it has been shown that it can occur (Baur et al., 2001). Our results indicate that the 4q subtelomere specifically would be more subject to any TPE mechanisms that may operate at the nuclear periphery or envelope.

These results further demonstrate a precedent for a reproducible spatial orientation for different parts of an individual human chromosome, with the large 4q arm pointing to the nuclear envelope and the p arm projecting inward. Results indicate that this orientation reflects an inherent tethering of the 4q subtelomere to the nuclear periphery; the positioning resists perturbation even in the foreign environment of a mouse nucleus or when translocated to another chromosome. Tethering of a single chromosomal site has been suggested in Drosophila melanogaster (Marshall et al., 1996). Our results now define a small segment at the tip of human 4q35 that appears to bear some sequence (which is not D4Z4) with an affinity for the peripheral nuclear rim.

As diagrammed in Fig. 4, we find an intriguing correlation between this chromosomal organization and recent SAGE data showing clustered expression along the chromosome, which does not merely reflect gene density (Versteeg et al., 2003; http://bioinfo.amc.uva.nl/HTMseq). Although the radial distribution of chromosomes may correlate with overall gene density (Boyle et al., 2001; Cremer and Cremer, 2001; Carmo-Fonseca, 2002), this does not explain the 4q versus 4p organization, which have similar gene densities despite marked differences in nuclear organization. However, the gene expression profile along the chromosome (Fig. 4 B) reveals the 4q subtelomeric region is substantially expression poor compared with 4p. Interestingly, Versteeg et al. (2003) identify chromosome 17q as a region of increased density of gene expression, which correlates well with our finding that 17q associates with SC35 domains. We interpret these results in light of a growing appreciation that the regulation of gene expression is controlled on multiple levels, one of which involves nuclear compartmentalization that can either promote or hinder transcription (Cockell and Gasser, 1999; Isogai and Tjian, 2003).

It has been speculated that the FSHD locus may be associated with heterochromatin and be subject to some form of chromosome position effect (Bickmore and van der Maarel, 2003). However, direct evidence had not been established, and one group, finding chromatin modifications similar to “inactive euchromatin” rather than constitutive heterochromatin, challenged this view (Jiang et al., 2003; Yang et al., 2004). Our findings demonstrate that the 4q35 region is clearly localized to a discrete nuclear “neighborhood” rich in heterochromatin, one of few human telomeres at the nuclear periphery. This close spatial association with heterochromatin, together with other evidence of a link to gene repression (Gabellini et al., 2002) and methylation (van Overveld et al., 2003) supports the hypothesis that D4Z4 at 4q35 is likely involved in regulation of the heterochromatic state.

Having demonstrated 4q35 is with the heterochromatic compartment normally, one mechanism by which D4Z4 deletion could influence gene expression entails relocation of the region away from this repressive compartment. Defects in nuclear organization have been widely speculated for FSHD (Bickmore and van der Maarel, 2003; Winokur et al., 2003). We tested this hypothesis by examining 4q organization in patient fibroblasts, myoblasts, and myotubes. The mutant allele showed no clear and consistent breakdown in 4q organization, demonstrating that a critical number of D4Z4 repeats is not required for tethering 4q35 to the nuclear periphery. Although we cannot completely rule out that more subtle mislocalization or changes in a very small subset of FSHD myoblasts could be significant for disease pathogenesis, small differences between myoblasts lines likely reflect normal biological variation. For example, more senescent cells (normal WI38 and GM17725) may correlate with more internal 4q alleles. It has been shown that senescence affects telomere integrity (Karlseder et al.,
and, interestingly, chromosome 4 contains the highest proportion of senescence-responsive genes (Chen et al., 2004).

Gabellini et al. (2002) provided evidence that genes (FRG1, FRG2, and ANTI) up to a few megabases away are up-regulated in FSHD muscle cells, which is consistent with D4Z4 normally functioning to repress gene expression over long distances. The idea that D4Z4 at 4q influences the heterochromatic state is further supported by a recent report that certain sites on the mutant allele are hypomethylated (van Overveld et al., 2003). Given our finding that D4Z4 contraction exhibits its effect on FSHD cells despite remaining in the heterochromatic neighborhood, this tandem repeat likely influences the heterochromatic state on a more local scale. Importantly, yeast studies demonstrate that genes can be expressed from the heterochromatic periphery; however, specific mechanisms are required to regulate expression in this repressive chromatin environment (Hediger and Gasser, 2002). For example, D4Z4 repeats could function akin to a silencer element, possibly at the transition between euchromatin and heterochromatin. In yeast, loss of a silencer element prevents propagation of heterochromatin and silencing that would otherwise occur at telomeres associated with the nuclear envelope (Feuerbach et al., 2002). Although some tandemly repeated sequences can form heterochromatin, protosilencers do not silence on their own. They propagate or maintain a heterochromatic state initiated at a silencer and typically require a specific copy number of tandem repeats for proper function (Fourel et al., 2002), similar to D4Z4 in FSHD.

However, results from Gabellini et al. (2002) have become controversial because two recent papers contest the expression data of these 4q35 genes (Jiang et al., 2003; Winokur et al., 2003). Likewise, an earlier paper concluded that the methylation status did not change in mutant alleles (Tsien et al., 2001) in contrast to recent findings (van Overveld et al., 2003). Cell-to-cell variability and duplicated loci on other chromosomes may obscure expression from 4q; ultimately, the most definitive way to demonstrate a potential cis-effect on FSHD cells is to compare expression in fibroblasts and muscle cells. Moreover, cell type and the precise mechanism mediating gene expression at 4q35 remain contentious. It is possible that the expression of genes of 4q35 is influenced by the local chromatin environment influenced by D4Z4 repeats. Plasmid pLAM containing the 3.3-Kb D4Z4 sequence was used to confirm the presence of four D4Z4 repeats. Plasmid pLAM containing the 3.3-Kb D4Z4 sequence was a gift of S. Winokur (University of California, Irvine, Irvine, CA; Hewitt et al., 1999).

Clear evidence favors a normal role for D4Z4 in maintaining gene repression; however, a given sequence can have a dual role in silencing or insulating from silencing (Kuhn and Geyer, 2003). Although speculative, the possibility merits consideration that D4Z4 can normally insulate neighboring genes from repressive heterochromatin for two reasons. First, this would fit with the recent finding that D4Z4 contraction only results in disease when present on the same chromosome as a polymorphic β satellite sequence (on half of normal alleles and all disease alleles; Lemmers et al., 2002). This would be explained if the presence of the β satellite augmented the heterochromatic signal, requiring the higher number of D4Z4 repeats to “insulate” genes in the vicinity of a stronger repressive signal. Second, we suggest that a role as transition or insulator element is implied by the singular organization of D4Z4 repeats on human chromosomes, showing a remarkable positioning between β satellite sequences and the very active rDNA genes on all acrocentric chromosomes (Fig. 7 A; Winokur et al., 1996).

Similarly, gene expression in1q21 would likely require insulation from the exceptionally large block of heterochromatin in 1q12; D4Z4 repeats lie at the interface of this heterochromatin–euchromatin boundary (Fig. 7 A and Fig. 5 A; nucleus).

These observations point to a possible functional relevance for a subtelomeric repeat family, about which little is known. Notably, we find that other D4Z4-associated loci have an unusual affinity for the nuclear or nucleolar periphery and reside between regions of euchromatin and β satellite DNA. Although the peripheral localization is most striking for 4q35, the strong commonality in nuclear organization of other D4Z4 loci raises the possibility of a common structural and/or functional role (with heterochromatin) for this subtelomeric repeat family.

Finally, our findings support earlier suggestions that telomeres are anchored (Luderus et al., 1996; Weipolsthammer et al., 1999; Nagele et al., 2001) but goes further to indicate that specific telomeres tether to specific intranuclear locations. Given that our results demonstrate tethering of 4q35 and also raise the possibility of a role for D4Z4 as an insulator, it would be interesting to examine the relationship to CTCF, a protein which has recently been reported to have a role in both insulating and tethering of specific sequences to the nuclear matrix and the nucleolar periphery (Yusufzai et al., 2004). This is but one of several important areas for future directions raised by this work.

Materials and methods

Probes, in situ hybridization, and immunolocalization

BACs used in this study, RP11-597P9 (4q35.1), RP11-279K24 (4q35.1), RP11-2H3 (4p16.3), RP11-288G11 (10q26.3), and RP11-509C1 (Xp21.1), were supplied by M. Rocchi (University of Bari, Bari, Italy), as was the plasmid p41n1/4 containing 4 alphoid centromeric sequences. RP11-597P9 is 0.5 Mb distal to RP11-279K24 and 4 Mb proximal to the D4Z4 repeats. Plasmid pLAM containing the 3.3-Kb D4Z4 sequence was a gift of S. Winokur (University of California, Irvine, Irvine, CA; Hewitt et al., 1999).

Conditions for probe labeling by nick-translation and in situ hybridization to PFA-fixed, Triton-extracted cells have been described previously (Tam et al., 2002). In addition to probes nick-translated with biotin or digoxigenin, prelabeled probes to 1p7, 1p7q, and 4q subtelomeres (Vysis Inc.) as well as a 18 bp PNA probe to (TTAGGG)n (Applied Biosystems) were used according to the manufacturer’s recommendations. For detection of general nuclear hnRNA, nonadenatured cells were hybridized with a probe against human Cot-1 RNA, as described in Hall et al. (2002).

To demarcate nuclear structures, the following antibodies were used: mouse anti-fibrillarin (gift of M. Pollard, Scripps Research Institute, La Jolla, CA), mouse anti-SC35 (Sigma-Aldrich), rabbit anti-lamin (Cell Signaling), and mouse anti-nucleopore (Affinity BioReagents, Inc.). Nuclear contour was also evident by DAPI DNA staining.

Cell lines and culture conditions

Lymphoblastoid FSHD cell lines were provided by S. Winokur and cultured in RPMI, 10% FBS, in a 37°C incubator with 5% CO₂. WI38 fibroblasts (American Type Culture Collection) were cultured in DME, 10% FBS. 50MB-1 normal myoblasts were a gift from H. Blau (Stanford University, Stanford, CA). Four FSHD myoblast cell lines (GM17725, GM17731, GM17869A, and GM17899) were obtained from the Coriell Cell Repository, from patient biopsies deposited by M. Ehrlich (Tulane University, New Orleans, LA), and characterized in our lab for myogenesis (see Online supplemental material). Myoblast cell lines were cultured in SKGM media (Clonetics) and for differentiation were shifted to DME (GIBCO BRL), 2% horse serum, upon reaching 80% confluency. A mouse somatic cell hybrid line, GM11687A (Coriell Cell Repository), was cultured in Ham’s F12:DME, 10% FBS. A I(X4) translocation cell line, GM11025 (Coriell Cell Repository), was grown in RPMI, 10% FBS.
Microscopy and image analysis

Single plane images and z-stacks [step size of 100 nm] were acquired using a microscope [model Axiovert 200; Carl Zeiss MicroImaging, Inc.] equipped with a 100× PlanApo objective [NA 1.4]. The Chroma 83000 multiband pass dichroic and emission filter set [Chroma Technology Corp.] was used with its excitation filters set up in a wheel to prevent optical shift. Triple-labeled experiments using FITC, Texas red, and Alexa 350 were mounted in Vectashield [Vector Laboratories]. Between 50 and 400 cells were examined directly through the microscope over two or more replicates for each experiment, as further specified in the figure legends. Images were captured on a camera [model Orca-ER; Hamamatsu]. Z-stacks were processed using constrained iterative deconvolution in Axiovision 4.1 or the retinex hue algorithm in Metamorph 4.6, and displayed as maximum value projections.

Online supplemental material

Characterization of four FSHD myoblast cell lines (including Figs. S1 and S2). Fig. S3 shows a description of nuclear volume calculations. Video 1 shows the distribution in three dimensions of total telomeres [red] in relation to nucleopores [green] and nucleoli [blue] in muscle (50MB-1). This video is a though-focus series of 40 planes spaced 100 nm apart, displayed at 10 frames per second. Video 2 shows a three-dimensional organization of 4q35 [red], D4Z4 [green], and fibrillar [blue] in muscle nuclei (50MB-1). This video is a through-focus series of 50 planes spaced 100 nm apart, displayed at 10 frames per second.

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Note added in proof. While this paper was in production a paper on a similar topic was published by Masny et al. [Masny, P.S., U. Bengtsson, S.A. Comings, D.E. 1980. Arrangement of chromatin in the nucleus. Hum. Genet. 53: 131–143.

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