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Repositioning of Muscle-specific Genes Relative to the Periphery of SC-35 Domains during Skeletal Myogenesis

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Previous studies have shown that in a given cell type, certain active genes associate with SC-35 domains, nuclear regions rich in RNA metabolic factors and excluded from heterochromatin. This organization is not seen for all active genes; therefore, it is important to determine whether and when this locus-specific organization arises during development and differentiation of specific cell types. Here, we investigate whether gene organization relative to SC-35 domains is cell type specific by following several muscle and nonmuscle genes in human fibroblasts, committed but proliferative myoblasts, and terminally differentiated muscle. Although no change was seen for other loci, two muscle genes (*Human β-cardiac* myosin heavy chain and *myogenin*) became localized to the periphery of an SC-35 domain in terminally differentiated muscle nuclei, but not in proliferative myoblasts or in fibroblasts. There was no apparent change in gene localization relative to either the chromosome territory or the heterochromatic compartment; thus, the gene repositioning seemed to occur specifically with respect to SC-35 domains. This gene relocation adjacent to a prominent SC-35 domain was recapitulated in mouse 3T3 cells induced into myogenesis by introduction of MyoD. Results demonstrate a cell type-specific reorganization of specific developmentally regulated loci relative to large domains of RNA metabolic factors, which may facilitate developmental regulation of genome expression.

INTRODUCTION

Formation of distinct cell types during the development of higher organisms requires the orchestrated expression of ~40,000 genes. Cells become progressively more restricted in developmental potential as they commit to specific lineages, with the result that only a small subset of genes is fully competent to be expressed in subsequent cell generations. Concomitant with this genome-wide epigenetic regulation are overall changes in the morphology and internal appearance of the cell nucleus, as exemplified by the cell type-specific arrangement of heterochromatin and of nucleoli (Comings, 1968; Manuelidis, 1984; Carter *et al*., 1991; Cremer and Cremer, 2001). The known clustering of gene-rich DNA into metaphase chromosome bands provides evidence of some higher order organization of the genome (Bickmore and Sumner, 1989; Saitoh and Laemmli, 1994). These and other observations have contributed to long-standing speculation that there may be a cell type-specific organization of genes in interphase nuclei that facilitates cell type-specific gene expression. Higher order organization may impact a gene's relationship to other intranuclear structures: for example, genes may have specific spatial arrangements relative to domains enriched in RNA metabolic factors (Xing *et al*., 1993; Xing *et al*., 1995; Smith *et al*., 1999), relative to the interphase chromosome territory and interchromosomal regions (Clemson *et al*., 1996; Kurz *et al*., 1996; Cremer and Cremer, 2001; reviewed in Mahy *et al*., 2002), or relative to heterochromatin (Xing *et al*., 1995; Brown *et al*., 1997; reviewed in Gasser, 2001).

The primary focus of this study is to compare the organization of specific genes relative to SC-35–enriched domains in the context of myoblast differentiation. SC-35 domains are subnuclear compartments rich in poly A RNA (Carter *et al*., 1991; Visa *et al*., 1993) and certain mRNAs (Shopland *et al*., 2002), as well as a variety of RNA metabolic factors, including spliceosome assembly factor SC-35 (Fu and Maniatis, 1990; Blencowe *et al*., 1994), numerous other splicing factors (Nyman *et al*., 1986; Spector *et al*., 1991), a subset of poly (A) polymerase (Schul *et al*., 1998), a putative RNA helicase (Gee *et al*., 1997), and a hyperphosphorylated form of RNA polymerase II (Bregman *et al*., 1995). These 20–40 globular domains consist of the more prominent (0.5–3 μm in diameter) components of the small nuclear ribonucleoprotein "speckled" staining pattern and occupy <5% of the nuclear volume, lying in a narrow focal plane in cultured human fibroblasts and myoblasts (Carter *et al*., 1993).

Quantitative analyses have shown that SC-35 domains have statistically highly significant associations with certain active genes and their transcripts (Xing *et al*., 1995; Smith *et al*., 1999). To date, of the multiple active genes examined this way, more than half have been shown to be highly (70–100%) associated with SC-35 domains, whereas inactive genes show only low (10–20%) apparent association. Clearly, the distribution of individual genes or pre-mRNAs

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relative to these domains is not random, but gene specific, with associated genes consistently positioning at the domain edge. Several inactive genes studied were frequently at the nuclear periphery, a region devoid of SC-35 domains and enriched with heterochromatin (Xing *et al*., 1995; Smith *et al*., 1999). These findings suggest the fundamental hypothesis of a cell type-specific genomic organization that provides many active genes immediate access to copious supplies of RNA metabolic factors within "domains," potentially facilitating expression of those genes.

Although indirect evidence suggests that association with SC-35 domains is related to gene expression, the relationship is not simple. For example, some genes producing highly spliced pre-mRNAs essentially never associate (Smith *et al*., 1999), and even genes that associate with high frequency are not always associated with a domain even while still clearly expressing pre-mRNA (Xing *et al*., 1993, 1995). Other work has shown that an intronless heat-shock gene associates with SC-35 domains upon heat shock, indicating that the association is not always related to splicing but may be related to transcription (Jolly *et al*., 1999). Although a relationship of this gene arrangement to cell type is implicated by some observations, to establish this key point requires a systematic analysis of several active and inactive genes relative to SC-35 domains in different cell types of a developmental system. Hence, it is important to track potential changes in the spatial relationship with SC-35 domains of several loci as they progress through developmental transitions and states of expression. Furthermore, it would be valuable to include analysis of one or more genes that are not particularly highly expressed and complex, to discriminate between changes in gene organization relative to SC-35 domains and the accumulation of factors on abundant nuclear RNA of a single gene.

To address the hypothesis that higher order organization of genes relative to SC-35 domains changes with development and cell type-specific gene expression, we investigated this for seven endogenous loci, including three muscle-specific genes, during in vitro skeletal muscle differentiation. Because changes in gene organization might occur concomitant with cell-type commitment, but before gene expression in a fully differentiated cell, we compared organization of several genes in differentiated muscle cells with that of both fibroblasts and committed but undifferentiated myoblasts. To further establish that observed changes in organization of specific genes with SC-35 domains was related to cell-type changes during myogenesis, we tested whether this reorganization could be recapitulated in mouse 3T3 cells induced to form muscle by introduction of MyoD. Finally, we briefly examined changes in gene organization with SC-35 domains in the context of other potential organizational changes, specifically the placement within the chromosome territory and localization relative to centric heterochromatin.

MATERIALS AND METHODS

Cell Culture and In Vitro Myogenic Differentiation

Normal human fibroblast cells (Detroit 551 or WI-38; American Type Culture Collection, Manassas, VA) were used as nonmyogenic cell controls, and were cultured as described previously (Xing *et al*., 1995). Flow-sorted undifferentiated normal human myoblast cells (strain 50Mb-1), isolated from the vastus lateralis muscle of a 10-year-old male, were kindly provided by Dr. Helen Blau (Stanford University, Palo Alto, CA) (Webster *et al*., 1988; Smith *et al*., 1999). Proliferative myoblast cells were cultured in growth media (GM) comprised of Ham's F-10 media supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% (vol/vol) chick embryo extract (60-Å ultrafiltrate), with penicillin and streptomycin, and seeded onto glass coverslips (Corning Glassworks, Corning, NY). In vitro myogenic differentiation was induced by culturing myoblasts in GM to ~70% confluence, and then switching to low serum differentiation media (DM; DMEM-low glucose supplemented with 2% horse serum (Hyclone Laboratories), 1 mM insulin, 1 mM dexamethasone (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin). Multinucleated myofibers typically were first noted on day 3, and differentiation was typically complete by day 7. Cell processing for in situ hybridization, involving detergent permeabilization and paraformaldehyde fixation, was as described previously (Xing *et al*., 1995).

A second cell differentiation model utilized mouse NIH3T3-derived cells (B22 cells) that had been induced to form myoblasts using a *MyoD*-expressing retrovirus, as previously described (Novitch *et al*., 1996). Cultures at ~50% confluency were infected for 30 h with one-third of the supernatant from a confluent 100-mm plate of BOSC23 cells generating *MyoD* retrovirus and polybrene (4 μg/ml) diluted into fresh media. Mock-infected plates were exposed to media and polybrene only. Differentiation was induced by growth in low serum medium for 36 h or more (Novitch *et al*., 1996).

The B22 cell line expresses dominant-negative BRG1 protein under the control of the tet promoter (de La Serna *et al*., 2000). For analysis of normal cells, B22 cells were grown in tetracycline, which represses expression of the mutant protein. To prevent myogenic gene expression, cells were grown without tetracycline, which allows expression of mutant BRG1. The dominant-negative BRG1 is ATPase deficient and forms dominant-negative SWI/SNF complexes that interfere with MyoD-induced differentiation (de La Serna *et al*., 2001a)

Hybridization Probes and Immunofluorescence Reagents

Human β-cardiac myosin heavy chain (*MyHC*) was detected using a 12.3-kb clone (p8-1A) from L. Leinwand (University of Colorado, Boulder, CO) (Liew *et al*., 1990) encompassing the intergenic spacer and extending partly into the 5′ region of the closely linked α-gene. The closely linked α- and β-genes are highly homologous, but only β is expressed in cultured muscle (Yamauchi-Takihara *et al*., 1989). We used a 30-kb cosmid clone containing the entire *MyoD* gene (cHMD-13) and 5′-upstream sequences (Goldhamer *et al*., 1992) from C. Emerson (University of Pennsylvania Medical Center, Philadelphia, PA). Human myogenin (*myf-4*) was detected using a 5.8-kb plasmid clone containing the entire human gene (Braun *et al*., 1989), supplied by H. Arnold (Technische Universität Braunschweig, Braunschweig, Germany). Mouse myogenin (Edmondson *et al*., 1992) was detected with a 16-kb genomic probe from D. Edmondson and E. Olson (University of Texas, M.D. Anderson Cancer Center, Houston, TX). Nonmyogenic sequences used were described previously (Xing *et al*., 1995). Whole chromosome 14 was detected using a digoxigenin-labeled Coatasome total chromosome probe (Oncor, Gaithersburg, MD).

A mouse monoclonal antibody against the SR (arginine/serine) spliceosome assembly protein SC-35 (Fu and Maniatis, 1990) was obtained from X.-D. Fu (University of California, San Diego, San Diego, CA) or Sigma-Aldrich (St. Louis, MO). SRM300, a 300-kDa SR-related nuclear matrix antigen used to detect SC-35 domains in mouse cells was provided by Dr. B.J. Blencowe (University of Toronto, Toronto, ON, Canada). Mouse anti-myogenin was purchased from BD PharMingen (San Diego, CA) and rabbit polyclonal IgG anti-*MyoD* was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Fluorescence In Situ Hybridization

Methods used here have been published in detail elsewhere (Carter *et al*., 1991; Johnson *et al*., 1991; Tam *et al*., 2002). In brief, DNA probes were nick translated with either biotin-11-dUTP or digoxigenin-16-dUTP (Roche Diagnostics, Indianapolis, IN). DNA-specific hybridization was accomplished by simultaneously hydrolyzing nuclear RNAs and denaturing target DNAs in 0.07 N NaOH for 5 min, followed by application of probe. RNA-specific hybridization involved applying probe to nondenatured cells such that cellular DNA is not available for hybridization. Cells subjected to immunofluorescence analysis after hybridization were fixed for 10 min before application of antibody. Total nuclear DNA was stained using 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 30 s, which in mouse preferentially stains the A-T–rich DNA present in all mouse centromeric heterochromatin. The chromosome 14 interphase territory was painted, as described previously (Clemson *et al*., 1996). Cells were permeabilized with 0.5% Triton X-100 for 7 min before a heat denaturation in 70% formamide, 2 × SSC at 85°C for 5 min. Ten microliters of whole chromosome paint, denatured for 5 min at 75°C and prehybridized at 37°C for 2 h, was applied to dehydrated cells, and hybridized overnight. The painting probe was detected using rhodamine antidigoxigenin antibody (Roche Diagnostics).

Microscopy and Analysis

An Axioplan microscope (Carl Zeiss, Thornwood, NY) designed for imaging multicolor fluorescence was used. Variable excitation using a filter wheel (Ludl Electronic Products, Hawthorne, NY) in conjunction with multiband-pass emission filters (Chroma Technology, Brattleboro, VT) was used for two or three color in-register imaging. High-resolution digital image capture was performed using a Photometrics 200 Series camera (Photometrics, Tucson, AZ), a high-resolution, low-depth of field objective (100×, numerical aperture 1.4; Carl Zeiss), and image acquisition and analysis software (GW Hannaway

and Associates, Boulder, CO; and MetaMorph, Universal Imaging, Dowingtown, PA).

Signal was scored as associated with an SC-35 domain when there was no visible separation between the signal and the SC35 domain boundary. Signal was occasionally associated with small concentrations of SC-35 (<0.5 μm), but this was not scored as associated with a domain unless a large (typically 0.5–3 μm) prominent domain was present. All experiments were viewed and, when possible, analyzed directly through the microscope. Data represents multiple experiments analyzed independently by at least two investigators. For analysis of association with the peripheral heterochromatin, gene signals were scored as associated if they were in contact with or very close (<0.6 μm) to the edge of the nucleus.

RESULTS

Localization of Muscle-specific and Nonmuscle-specific Sequences in Myotube Nuclei

Before comparing the distribution of genes in different cell types, we first examined the distribution of seven different sequences, including muscle and nonmuscle genes, in fully differentiated human skeletal muscle, by using fluorescence in situ hybridization to detect specific nuclear RNAs and/or the corresponding gene (see MATERIALS AND METHODS). In >98% of the postmitotic nuclei within multinucleated skeletal myotubes, the RNA accumulations from the β-cardiac myosin heavy chain genes (*cMyHC*) associate with SC-35 domains. As described in MATERIALS AND METHODS, any signal that seemed to contact the domain with no visible separation is scored as "associated," whereas even a slight visible separation is considered nonassociated. Typically, both of these slightly elongated accumulations overlap much of the domain (Figure 1, A and C), as described previously and termed a "type I" RNA pattern (Smith *et al*., 1999). The *cMyHC* gene, in contrast, consistently localizes immediately adjacent to or partially overlapping the edge of the SC-35 domain, similar to COL1A1 gene and its nuclear RNA (Xing *et al*., 1995).

The positions of two other muscle-specific genes, myogenin (*myf-4*) and *MyoD*, were also examined in differentiated myofibers. Human myogenin and *MyoD* are muscle regulatory proteins that are expressed at similar levels from genes of similar size and complexity, both of which are less complex and less highly expressed than *cMyHC* (Braun *et al*., 1990; Goldhamer *et al*., 1992). In contrast to the more globular or track-like morphology of *cMyHC* RNA, myogenin and *MyoD* nuclear RNAs produced two small bright foci (Figure 1, B, D, and G), similar to the round hybridization spots from a single copy gene. For >200 myogenin RNA foci examined, 88% were associated with a prominent (0.5–3.0 μm in diameter) SC-35 domain (Figure 1, B and D). The domain was much larger than the round spot of RNA signal, which contacted or slightly overlapped the domain periphery but did not occupy its central regions. This RNA distribution pattern has been termed "type II" (Smith *et al*., 1999). Because the RNA foci are smaller and at the edge of the domain, the domain clearly represents a distinct entity, a large accumulation of factors with which the gene and its RNA associates. Hybridization to myogenin DNA yielded results comparable with the RNA, with the gene also localizing to the domain periphery (Figure 1E). Note that in some nuclei both alleles associated with the same domain (Figure 1, E and F), supporting the idea that multiple genes cluster at these structures, as further demonstrated in Shopland *et al.* (2003).

In the same experiments, the *MyoD* RNA foci, virtually indistinguishable in size and intensity to myogenin RNA foci, did not associate with prominent splicing factor domains in the majority of myotube nuclei (Figure 1G, inset). In some experiments, *MyoD* RNA was associated with very small concentrations of SC-35, but only infrequently (12%) seemed to contact one of the 20–40 prominent (0.5–3.0 μm) SC-35 domains. These low rates of apparent association are in keeping with that expected for a "random" distribution within the limits of our analytical approach (Xing *et al*., 1995). Similar to its nuclear RNA, only 12% of *MyoD* DNA signals associated with SC-35 domains (Figure 1H). Hence, despite close similarities in the size of their genes and nuclear RNA accumulations, the myogenin gene is expressed at the periphery of a large domain of RNA metabolic factors, whereas the *MyoD* gene is not. These results support our earlier suggestion that genes are expressed in different nuclear environments, and are consistent with previous evidence that there are sufficient splicing factors in the interdomain space to process even highly complex pre-mRNAs (Smith *et al*., 1999).

For comparison to a broader view of sequence localization relative to SC-35 in muscle nuclei, we examined other nonmyogenic sequences in differentiated myotube nuclei, including sequences that are not expressed or do not encode pre-mRNA. As summarized in Figure 2, hybridization to the RNA polymerase III transcribed gene for human 5S rRNA (Little and Braaten, 1989) indicated only infrequent association with SC-35 domains. Similarly, the transcriptionally inactive human albumin gene (HSA-10; ATCC) (Figure 2), and a chromosome 17 alphoid repeat sequence, p3.6 (our unpublished data; Waye and Willard, 1986) revealed very low levels of apparent association, consistent with random expectation. These signals also frequently localized to a different focal plane of the nucleus than do the SC-35 domains and their associated genes (Figure 1, A–E) (Xing *et al*., 1993, 1995). An interesting case is that of collagen 1A1 RNA (*COL1A1*), previously shown to consistently associate with one of the largest SC-35 domains in fibroblast nuclei, a cell-type in which it is highly expressed (Xing *et al*., 1995). Because this gene is also expressed in myoblasts but downregulated to low or negligible levels after cells fuse to form postmitotic skeletal muscle (Beach *et al*., 1985), it was interesting to determine whether the gene would remain associated with an SC-35 domains. The myotube nuclei exhibited RNA foci much smaller in developing muscle fibers than in fibroblasts. However, these still associated with a prominent SC-35 domain, consistent with the domain representing a separate entity. More than 85% of collagen gene signals remained positioned at the periphery of a prominent domain in myotube nuclei, down just slightly from the ~99–100% association seen in fibroblasts (Xing *et al*., 1995) and below.

The above-mentioned results demonstrate a specific association of genes with SC-35 domains in muscle fibers, including genes that encode both a regulatory factor and more abundant structural proteins. These results also support that this spatial localization is locus specific and not a property of all genes expressing intron-containing RNAs, as discussed further below.

Are Genes That Localize with SC-35 Domains in Human Skeletal Muscle Organized in the Same Way within Human Diploid Fibroblasts?

For those muscle genes that are associated with SC-35 domains, is the association related to cell type and/or expression of that gene? Because *cMyHC* and myogenin genes associated at high frequency with domains in expressing muscle cells, we addressed whether the same spatial location of these genes was observed in other nonexpressing cell-types, e.g., possibly as a coincidence of chromosomal localization. To address this, we examined human diploid

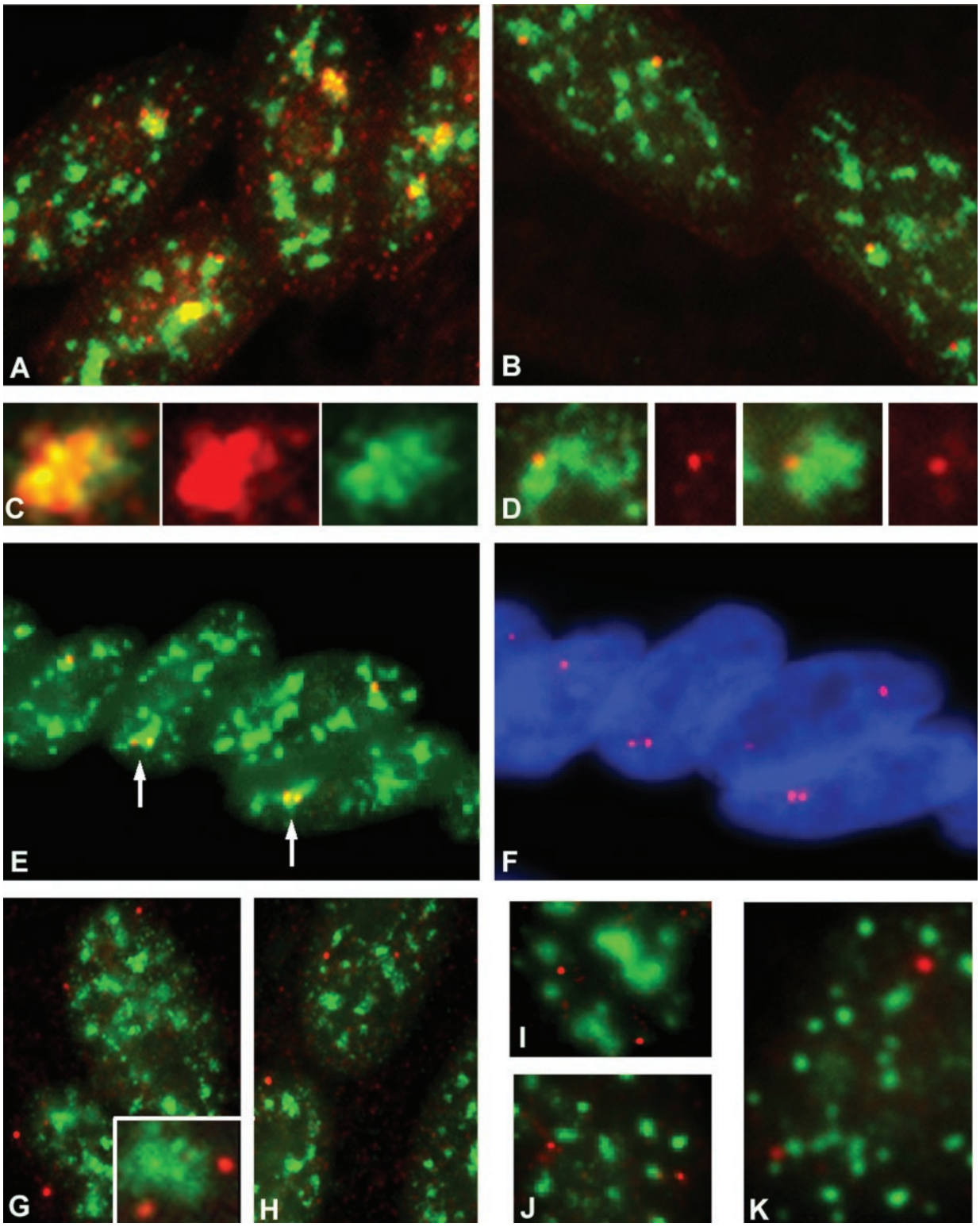

Figure 1. Detection of muscle-specific genes or transcripts (red) with SC-35 domains (green) in fully differentiated multinucleated myotubes (A–H) or in determined but undifferentiated myoblast cells (I, J, and K). (A and C) *cMyHC* RNA localizes with and frequently fills the interior regions of SC-35 domains. Areas of overlap are yellow. (C) Enlargement of a single *cMyHC* RNA track and SC-35 domain illustrating overlap (left-most panel). (B and D) Myogenin RNA associates with and localizes at the SC-35 domain periphery. (D) Enlargement of two domains illustrating specific and consistent association of myogenin RNA foci at the domain edge. Note the size of the myogenin RNA foci detected are approximately the size of a gene signal as seen in E and F and are considerably smaller than the domain. (E and F) Occasionally, both alleles of the transcriptionally active *myogenin* gene associate with the same SC-35 domain (arrows) in myotube nuclei. (F) The same myotube as in E, stained with DAPI (blue) to delineate multiple nuclei. (G and H) *MyoD* RNA (G) and DNA (H) do not associate with prominent SC-35 domains. Inset in G shows an enlarged view of the separation of RNA signal from the domain. (I–K) Myoblast cells were cropped to show enlargements of all three muscle-specific genes, *cMyHC* (I), *myogenin* (J), and *MyoD* (K), are not associated with SC-35 domains in myoblast cells before terminal differentiation.

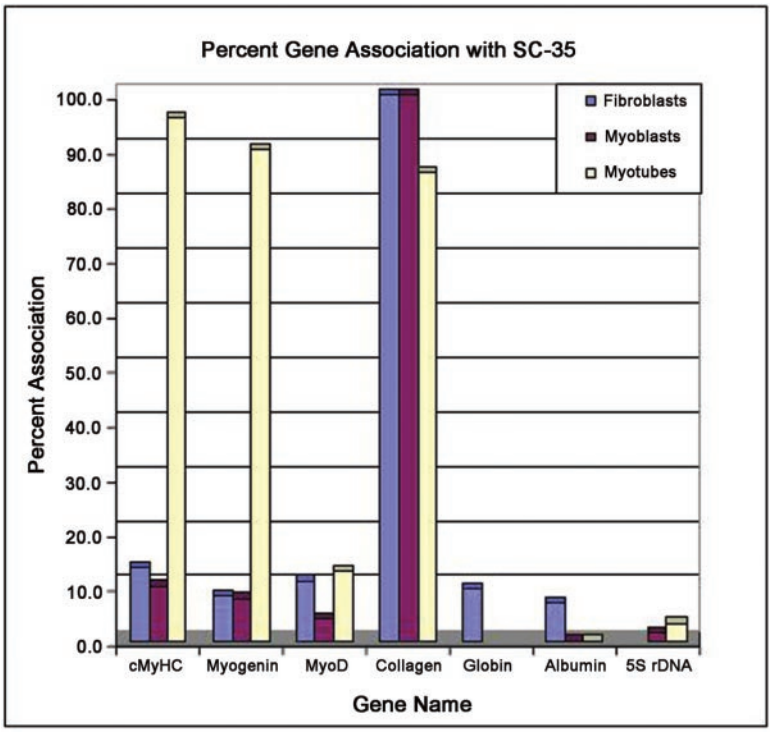

Figure 2. Quantitative analysis of gene organization in relation to myogenesis. The association of muscle-specific, nonmuscle, and nonexpressed sequences with SC-35 domains in differentiated myotubes, determined myoblasts, and nonmuscle fibroblast cells. Data were generated from DNA and/or RNA hybridizations. A sharp increase in association can be seen for *cMyHC* and *myogenin* in differentiated myotubes, concomitant with expression. No such change in association was seen for *MyoD* or for the nonmuscle-specific sequences. The data generated were gathered from examination of a minimum 50–200 signals, with the exception of albumin in myotubes where 10 signals were scored and all found to be nonassociated.

fibroblasts that neither express these genes nor are committed to do so.

In striking contrast to the gene localization observed in differentiated muscle, only 13% of *cMyHC* gene signals and 8% of myogenin gene signals seemed to contact an SC-35 domain in fibroblast cultures. The *MyoD* gene signal showed 11% association, similar to that seen in myotube nuclei (Figure 2). Hence, these results support that the *cMyHC* and myogenin genes do not associate with domains in this nonmyogenic cell-type, but become specifically positioned at the domain periphery in nuclei of differentiated muscle. To rule out that this was due to some global rearrangement of the nucleus upon muscle differentiation, four other nonmyogenic sequences examined in muscle cells were reexamined here. As shown in Figure 2, none of these showed a change in their relationship to the SC-35 domains; only the expressed *cMyHC* and myogenin genes showed this change in position. Hence, these results demonstrate that association of specific genes with domains is cell type dependent, occurring in differentiated cells that express the genes but not in nonmyogenic cells that do not.

Does Localization with SC-35 Domains Correlate with Muscle Differentiation or Myoblast Commitment to the Myoblast Lineage?

At what point in development of specific cell types does the difference in nuclear gene position between muscle and fibroblasts arise? Is terminal differentiation necessary or might the genes become positioned adjacent to an SC-35 domain during commitment to a myoblast lineage, e.g., in cells determined to form muscle but that have not yet differentiated and expressed these genes. To address this, sequences were assayed in committed but undifferentiated satellite myoblasts, still in proliferative phase. Both nonexpressed *cMyHC* and myogenin genes showed very little (<12%) association with SC-35 domains, not significantly different from random expectation (Figures 1, I and J, and 2). *MyoD*, even though expressed early in satellite myoblasts grown in culture, (Smith *et al*., 1994; de La Serna *et al*., 2001a), did not preferentially associate, in keeping with its localization to nondomain space in myotubes (Figure 1K). The frequency of association for the nonmyogenic sequences was very similar to that in fibroblasts. A summary table of all results is presented in Figure 2. These results show that these genes are not prepositioned in myoblasts that are still proliferative and nonexpressing. Hence, developmental commitment to a muscle lineage does not itself result in repositioning of muscle genes relative to factor rich SC-35 domains.

To further establish a link between splicing factor domain association and changes in gene expression during muscle differentiation, we examined the myogenin gene in a different model system, using mouse NIH3T3-derived B22 cells induced to differentiate to muscle by introduction of a retrovirus expressing *MyoD*. It has been previously documented that expression of this master muscle regulatory factor can "convert" nonmuscle cells to a muscle pathway and induce muscle-specific gene expression, including myogenin (Davis *et al*., 1987; Novitch *et al*., 1996). As shown previously for both *cMyHC* and myogenin (de La Serna *et al*., 2001a), introduction of *MyoD* into the B22 cells converts them to myoblasts which, when cultured under differentiation-promoting low serum conditions (see MATERIALS AND METHODS), withdraw from the cell cycle and express muscle-specific genes (in cells that remain mononucleated). The expression of muscle markers was confirmed using both Western blots (our unpublished data) and immunofluorescence (Figure 3E). In several experiments the frequency of cells expressing *MyoD* or myogenin was typically ~70%. In the mock-infected, nonmuscle cells on average 19% of mouse myogenin gene loci seemed to contact an SC-35 domain (Figure 3, A and B). This is in keeping with a "random" apparent association, but may be made slightly higher because the B22 cells tend to have smaller but more numerous

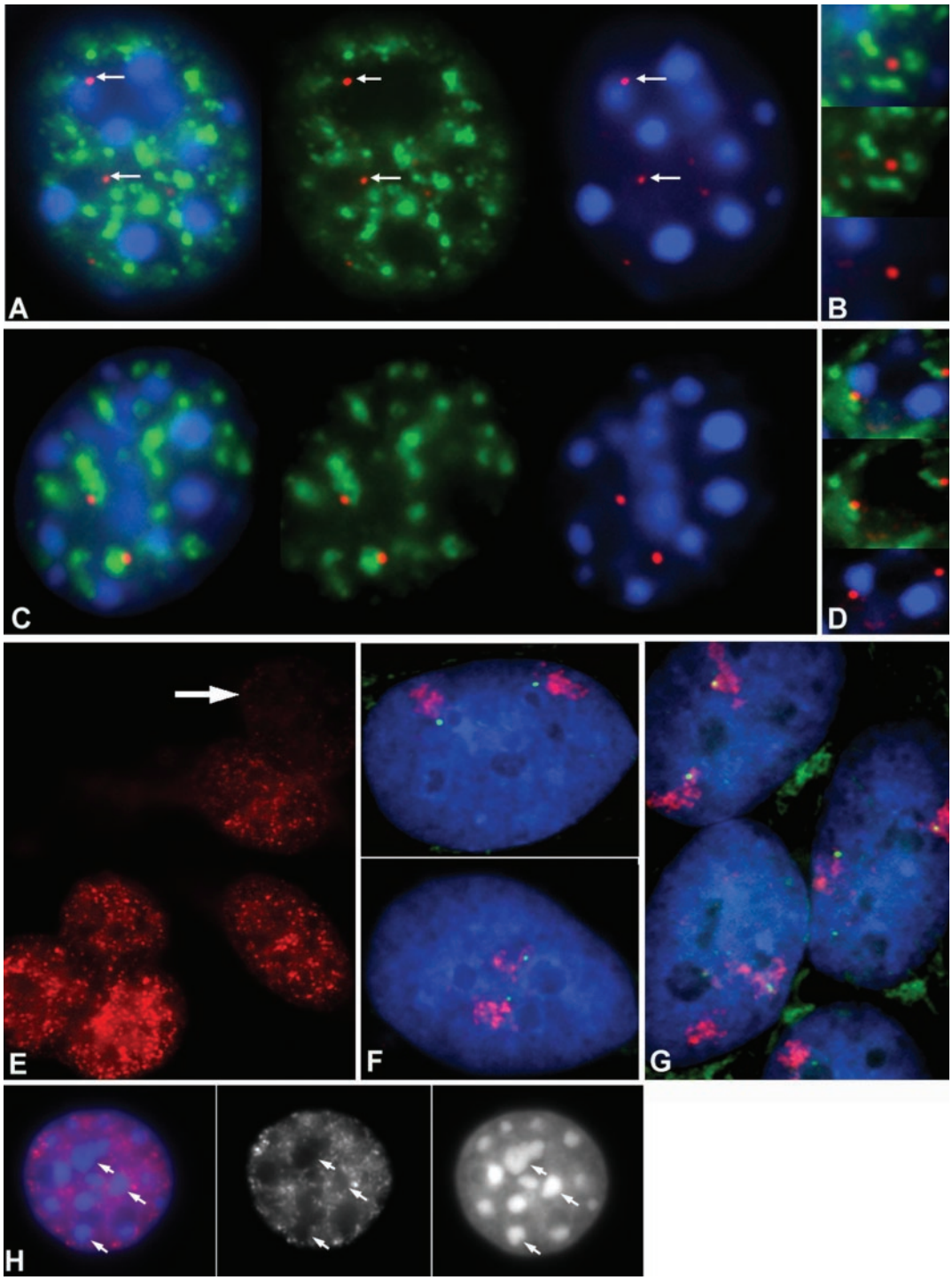

Figure 3. Localization of muscle specific genes relative to SC-35 domains, centric heterochromatin, and chromosome territories in undifferentiated and differentiated mouse NIH3T3 (B22) (A–E and H) or human (F and G) fibroblast cells. (A–D) Colors were separated to allow distinct visualization of the mouse *myogenin* gene signal (red) with SC-35 domains (green) and DAPI staining of mouse centromeric heterochromatin (blue). (A and B) The mouse *myogenin* gene does not associate with domains in undifferentiated cells. (C and D) The mouse *myogenin* gene in *MyoD*-induced cells shows a change in association pattern such that the gene localizes to the edge of SC-35 domains, similar to that seen in human cells. The *myogenin* gene does not show a specific relationship to heterochromatin (blue) in either undifferentiated (A and B) or differentiated cells (C and D). The *myogenin* gene was occasionally associated with heterochromatin (A, right cell; upper left signal and left signal in D) despite differentiation status but was predominately unassociated. (E) Myogenin protein staining demonstrating frequency of differentiation (arrow points to one negative cell in a field of six). (F and G) Although certain muscle specific genes can change their position relative to SC-35 domains upon differentiation, the *cMyHC* gene localizes at the outer boundaries of its chromosome paint despite its expression status in both undifferentiated (F) and differentiated human myotubes (G). The peripheral localization was consistently apparent in two-dimensional images of whole cells. (H) Mouse NIH3T3 cells hybridized with hnRNA (red) by using a probe to Cot-1 DNA reveal regions of low hnRNA concentration in regions of DAPI-bright (blue) A-T–rich centromeres. A rim of heterochromatin is delineated by the absence of cot-1 RNA around the nuclear periphery.

SC-35 domains. Interestingly, in B22 cells induced to undergo myogenesis by *MyoD*, the SC-35 domains became fewer but larger, as seen in several experiments (supporting earlier observations in differentiating rat muscle; Lawrence *et al*., 1993). Because infection did not result in *MyoD* expression in 100% of cells, it was important to identify myogenin-expressing cells by hybridizing to myogenin nuclear RNA, which showed that the level of association in these cells was 89% (Figure 3, C and D). This is in keeping with our scoring of the association level of the myogenin gene in cultures with a ~70% infection rate. This fourfold or more increase in the localization of the myogenin gene with SC-35 domains was seen in numerous experiments, demonstrating that *MyoD*-induced differentiation of B22 cells largely recapitulates the repositioning of this muscle-specific gene adjacent to SC-35 domains.

Does Association with SC-35 Domains Require Transcriptional Activation?

We cannot rule out that these genes become "poised" adjacent to an SC-35 domain in a small fraction of postmitotic myoblasts just before their expression. However, results clearly demonstrate that repositioning of both alleles of myogenin and *cMyHC* relative to SC-35 domains typically occurs concomitant with or very close to initiation of mRNA expression.

The above-mentioned findings clearly demonstrate that there is a repositioning of two muscle specific genes relative to SC-35 domains concomitant with cell type differentiation and gene expression. The temporal resolution is sufficient to conclude that gene expression and domain association occur close in time during muscle differentiation. However, location of genes adjacent to an SC-35 domain may occur just before, at the same time, or just after gene expression begins. A priori, the most likely possibility may seem to be that active RNA synthesis and a sufficient level of RNA on the gene may be a prerequisite for this association to occur. However, the results mentioned above do not rule out the possibility that the gene becomes positioned with the domain as terminally differentiating cells withdraw from the cell cycle, but just before gene expression. Hence, the gene might become "poised" for expression during a narrow window of time.

Although this is a difficult question to address conclusively, we present initial results that suggest that active gene expression might not be required and that prepositioning of the gene before expression remains a viable possibility. Again, we used the same system as described above in which NIH3T3 cells are induced by *MyoD*, but in this case, we examined the consequences of inducible dominant negative expression of BRG1, which forms dominant-negative SWI/SNF, a chromatin remodeling complex that has been shown necessary for muscle-specific gene expression in these cells. Expression of the mutant SWI/SNF has been shown to inhibit expression of myogenin mRNA and protein to undetectable or negligible levels, while at the same time allowing withdrawal from the cell cycle (de La Serna *et al*., 2001b). We had anticipated that the interference with transcription would prevent any change in location of the myogenin gene relative to the SC35 domains after *MyoD* induction. Surprisingly, in duplicate experiments there was an increased association of the myogenin gene with an SC35 domain, significantly above the typical "background" random association of ~15–20% (average 19%) in mock infected cells (no *MyoD*). Myogenin gene association with an SC-35 domain in *MyoD*-infected cultures expressing the mutant SWI/SNF ranged from 34 to 51%. Because muscle-specific gene expression is inhibited in these cells, we could not use the presence of myogenin nuclear RNA foci to distinguish expressing cells from those that were not infected with *MyoD* or not effectively induced to express muscle genes, as in the above-mentioned experiments with normal SWI/SNF. Immunofluorescence analysis of parallel cell samples indicated that in these experiments the average *MyoD* infection rate was 55–60%, thus the frequency of SC35 association of 34–51% in the total population remains approximate but likely underrepresents the association rate in cells induced by *MyoD*.

Further analyses of this and other genes would need to be examined to conclude that gene expression is not required for gene relocalization relative to SC35 domains. However, these initial findings do indicate that domain association increases under conditions in which myogenin gene expression is repressed, although the extent of association may not be as great as it would otherwise be when RNA synthesis was more active. These results are sufficient to indicate that it should not be presumed that significant RNA synthesis is required. Rather, these results raise the possibility that *MyoD* induction of terminal differentiation, in the absence of significant RNA synthesis, can cause reorganization of certain muscle-specific genes proximal to factor-rich domains. In either case, what is most clear is that collectively our findings show a definitive reorganization of two muscle-specific genes adjacent to SC35 domains during a short window of time during which myoblasts differentiate into muscle, whereas no such nuclear repositioning for several other sequences occurred.

Does Gene Position Change within the Chromosome Territory or Relative to Centromeric Heterochromatin?

The above-mentioned results demonstrate that the cMyHC and myogenin genes become repositioned with cell type relative to SC-35 domains. We briefly examined positioning of these genes relative to other features of nuclear topography that have been implicated in gene expression, to assess whether these change concomitantly with SC-35 domain association. Previous evidence indicates that SC-35 domains most frequently reside between chromosome territories (Zirbel *et al*., 1993; Clemson and Lawrence, 1996) and that gene loci may localize specifically at the outer periphery of this territory. Hence, changes in position relative to the chromosome territory and interchromosome space might coincidentally impact position relative to SC-35 domains, or vice versa. Some evidence indicates that certain active genes position at the periphery of the territory (Dietzel *et al*., 1999; Volpi *et al*., 2000), whereas analysis of other genes indicates that they maintain a peripheral position irrespective of activity or cell type (Kurz *et al*., 1996; Dietzel *et al*., 1999). We examined the localization of the *cMyHC* gene relative to its chromosome 14 territory in both the active state in differentiated myotubes and the inactive state in myoblasts. As shown in Figure 3, F and G, the gene was clearly and consistently positioned at the outer boundary of the chromosome 14 territory in both cell types regardless of activity status. The percentage of *cMyHC* genes localized at or just internal to the territory periphery was 81 and 84%, respectively, for myotubes and myoblasts. There was no significant difference in the small percentage of signals, which seemed slightly separate from the chromosome territory. This confirms prior evidence that muscle genes are peripherally localized within the chromosome territory in different cell types (Kurz *et al*., 1996). Hence, the positioning of the *MyHC* gene adjacent to an SC-35 domain is not a consequence of a repositioning relative to the chromosome territory.

In recent years, there has been increasing interest in the idea that inactive genes may be specifically positioned with respect to centromeric or peripheral heterochromatin and that close association of certain genes with this heterochromatic compartment may causally inactivate their expression (Brown *et al*., 1997; Francastel *et al*., 1999). To examine the proximity of the myogenin gene to centric heterochromatin, we took advantage of the fact that the A-T–rich centromeres of mouse chromosomes are easily visualized with DAPI staining. That DAPI staining demarcated regions of inactive heterochromatin was confirmed by hybridization to heterogenous nuclear RNA (hnRNA) by using a probe to Cot-1 DNA, which was previously shown to delimit inactive heterochromatin (Hall *et al*., 2002). Figure 3H demonstrates that DAPI-bright A-T–rich centromeres occupy regions of low hnRNA concentration and further illustrates that these cells have a relatively thin rim of heterochromatin at the nuclear periphery. The association of the myogenin gene to centromeric heterochromatin was examined in expressing and nonexpressing B22 cultures, by using the model system described above. In non-*MyoD*–induced cell cultures in which no cells express the myogenin gene, 16% of the myogenin gene signals seemed associated with the centromeric heterochromatin (in contact at the periphery) (Figure 3, A and B). In *MyoD*-induced cultures, in which most cells express this gene, the frequency of association with centromeric DNA decreased slightly to 14% (Figure 3, C and D). Frequently, one allele in a nucleus would seem to contact heterochromatin and the other would not, consistent with a potentially random relationship (Figure 3D). Infrequently a gene seemed to contact both an SC-35 domain and centric heterochromatin simultaneously (Figure 3D). Furthermore, $<1\%$ of signals were found to be at the nuclear periphery. We conclude that the myogenin gene, in 3T3 cells that normally will never express muscle genes, is not preferentially positioned in a domain of centric heterochromatin, nor does its position relative to centric heterochromatin change upon induction, in contrast to the sharp increase in SC-35 domain association concomitant with *MyoD*-induced expression.

These results support that in differentiated cells *cMyHC* and myogenin genes reposition specifically with respect to SC-35 domains and that this localization is not a coincidental result of global DNA reorganization or changes in gene position relative to either the chromosome territory or, in this case, centric heterochromatin. Rather, these results indicate that all regions of the nuclear periphery or the broader euchromatic nucleoplasm are not equivalent and that a key parameter that distinguishes different nuclear regions is proximity to SC-35 domains.

DISCUSSION

This study demonstrates directly that higher level organization of specific genes relative to SC-35 domains is cell type specific and correlates with changes in gene expression during differentiation. This demonstration of cell type-specific gene positioning suggests that spatial organization relative to factor-rich domains may contribute to gene regulation during development. Epigenetic changes during development permanently impact and coordinate the expression of tens of thousands of genes. Current concepts of gene regulation focus largely on mechanisms that transiently impact expression of just one or a few genes. However, mechanisms that provide heritable, broad-scale genomic regulation may be more likely to involve stable structural changes to chromatin, not only at the level of nucleosome packaging and the 30-nm chromatin fiber, but potentially to the higher order arrangement of genes or chromosome segments within the nucleus. At its most basic level the growing recognition that the nucleus contains numerous "intranuclear compartments" means that the environment of different genes is different and therefore could influence gene expression; clearly, a gene's position adjacent to a large domain enriched in numerous RNA metabolic factors could reasonably be expected to facilitate production of its RNA.

Because association with SC-35–rich domains is neither invariant for a given gene (Xing *et al*., 1993) nor characteristic of all genes expressing complex pre-mRNAs (Smith *et al*., 1999), it was important to address directly whether associations seen for a given gene change with cell type-specific gene expression. Results presented here clearly demonstrate that the *cMyHC* and myogenin genes only become associated with SC-35 domains upon cell type differentiation. In addition to demonstrating cell type specificity, results here further strengthen earlier evidence that a substantial fraction of genes expressing spliced pre-mRNAs associate with SC-35 domains. In this regard, results for myogenin RNA are important because this muscle regulatory factor is not so highly expressed or highly complex as some of the other pre-mRNAs that have been shown to associate with SC-35 domains, such as *cMyHC*. Furthermore, the myogenin RNA focus is far smaller and peripheral to the SC-35 domain with which it typically associates; it therefore could not have "formed" the domain, but rather, the gene becomes associated with a domain of splicing factors that is a separate entity and likely existed before expression of this gene. As discussed in detail elsewhere (Smith *et al*., 1999; Johnson *et al*., 2000) several observations support that the domain is not just comprised of factors bound on an individual pre-mRNA, but a larger structural conglomerate of numerous factors with which individual gene loci do or do not associate.

Consistent with results of uridine incorporation studies (Fakan and Puvion, 1980; Jackson *et al*., 1993; Wansink *et al*., 1993), our earlier analyses of individual genes showed that complex pre-mRNAs can be transcribed and spliced by the less concentrated splicing factors dispersed through the nucleoplasm (Xing *et al*., 1993; Smith *et al*., 1999). Comparison of the myogenin and *MyoD* results is informative in this regard, because despite the similarity in complexity and abundance of these pre-mRNAs, myogenin typically associates with a discrete SC-35 domain, whereas the *MyoD* gene does not. What would account for this difference? As previously discussed (Smith *et al*., 1999), we suggest that whether or not a given type II gene associates with an SC-35 domain may depend not only on its expression but also on the presence of specific regulatory elements and/or upon the constraints of its chromosomal context. Hence, the myogenin gene may be on a region of a chromosome with greater access to these regions than the *MyoD* gene. Other studies of *Drosophila* nuclei suggest that genes have short-range lateral mobility, exhibiting "constrained diffusion" within a region $\sim 1\ \mu$m square (Marshall *et al*., 1997). Where SC-35 domains initially form is a distinct question, but we suggest this may be determined by the location of type I genes, such as *cMyHC* and *COL1A1*, whose particularly high demand for RNA metabolic factors might serve to nucleate a domain; similarly they might form at sites with a high density of active genes. Recently, we have found evidence that this relates to the organization of genes within individual chromosome bands (Shopland *et al*., 2003). Whatever the mechanism that determines where domains form, our results indicate an intimate, cell differentiation-specific relationship between specific chromosomal loci and SC-35 domains.

Whereas *cMyHC* and myogenin genes changed their spatial association with SC-35 domains concomitant with skeletal myogenesis, a parallel change in gene position relative to the chromosome territory or to centromeric heterochromatin was not seen. Consistent with earlier observations (Kurz *et al*., 1996), we observed a striking and consistent localization of the *cMyHC* gene at the periphery of the chromosome 14 territory in all cell types studied, irrespective of activity. Because SC-35 domains typically reside between chromosome territories, a priori it was possible that association with domains might coincidentally arise by repositioning of the gene to the periphery of the territory. Our results show that this is not the case, and make the point that all regions of the chromosome territory periphery are not equivalent, because only a small part of the periphery contacts an SC-35 domain. Even though both active and inactive c*MyHC* genes positioned at the chromosome territory periphery, it is only the active c*MyHC* gene that associates with SC35. It has been reported for the major histocompatibility gene complex (Volpi *et al*., 2000) that the locus becomes more peripheral by the DNA visibly extending or looping out upon activity. We did not observe such a change here; however, we cannot rule out that some very subtle change in extension of the *cMyHC* gene occurs upon expression.

In our initial analysis of active and inactive genes in the same cell type (Xing *et al*., 1995), we noted that inactive genes localized to heterochromatic regions at the nuclear or the nucleolar periphery, whereas active genes were with SC-35 domains (Xing *et al*., 1995), which themselves avoid the peripheral heterochromatic region (Carter *et al*., 1991; Carter *et al*., 1993). Because genes localized in a heterochromatic region are not associated with SC-35 domains, and vice versa (Xing *et al*., 1995), we suggest that these represent alternative nuclear compartments. However, this work further demonstrates an important point: that not all locations within the broader euchromatic compartment are equivalent, and an inactive gene does not necessarily have to position with regions concentrated with heterochromatin, as seen here for the inactive myogenin gene. This is consistent with very recent evidence that loci differ in their relationship to heterochromatic domains when inactive (Brown *et al*., 2001), analogous to our finding that individual loci differ in their association with SC-35 domains when active (Xing *et al*., 1995; Smith *et al*., 1999). A recent study of chromosomal DNA organization relative to these SC-35 domains supports the idea, further suggested here, that SC-35 domains provide focal centers for smaller "euchromatic neighborhoods" within the broader euchromatic compartment of the nucleus (Shopland *et al*., 2003). We believe these locus-specific differences in nuclear position clearly have functional implications: the consequent positioning of genes close to domains rich in RNA metabolic factors may facilitate expression of the corresponding RNAs, and similarly, positioning away from these domains may contribute to repression. Although our findings leave open the possibility that gene positioning precedes transcriptional activation, an important area of investigation in the future will be to establish whether the localization of a gene with domains requires full gene transcription or whether domain association actually contributes to gene expression by promoting the metabolism of pre-mRNAs.

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