Immunocytochemistry of nuclear domains and Emery-Dreifuss muscular dystrophy pathophysiology

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The present review summarizes recent cytochemical findings on the functional organization of the nuclear domains, with a particular emphasis on the relation between nuclear envelope-associated proteins and chromatin. Mutations in two nuclear envelope-associated proteins, emerin and lamin A/C cause the Emery-Dreifuss muscular dystrophy; the cellular pathology associated with the disease and the functional role of emerin and lamin A/C in muscle cells are not well established. On the other hand, a large body of evidence indicates that nuclear envelope-associated proteins are involved in tissue-specific gene regulation. Moreover, chromatin remodeling complexes trigger gene expression by utilizing the nuclear matrix-associated actin, which is known to interact with both emerin and lamin A/C. It is thus conceivable that altered expression of these nuclear envelope-associated proteins can account for an impairment of gene expression mainly during cell differentiation as suggested by recent experimental findings on the involvement of emerin in myogenesis. The possibility that Emery-Deifuss muscular dystrophy pathogenesis could involve alteration of the signaling pathway is considered.

Key words: Emery-Dreifuss muscular dystrophy, emerin, lamin A/C, nuclear envelope, chromatin, nuclear signaling, chromatin remodeling complexes, immunocytochemistry

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The finding that Emery-Dreifuss muscular dystrophy (EDMD) is genetically related to mutations in either emerin or lamin A/C has focused the attention on the actual involvement of the nuclear envelope in the pathophysiology of the disease. Mutations in lamin A/C gene have been implicated in several human diseases, including EDMD, limb-girdle muscular dystrophy type 1B, hyperthrophic cardiomyopathy, Dunnigan-type familial partial lipodystrophy, and axonal neuropathy Charcot-Marie-Tooth disorder type 2. These disorders have been named nuclear envelopathies, or laminopathies. The common origin of these diseases raises the importance of the functional role of lamin A/C and nuclear envelope-associated proteins in the maintenance and/or acquisition of the cell phenotype. The involvement of different nuclear domains in the control of developmental expression of tissue specific genes has been suggested. However, the functional features of these domains as related to nuclear envelope-associated protein expression and/or localization are yet not defined. A possible functional link among chromatin remodeling complexes, intra-nuclear signaling pathways and nuclear envelope-associated proteins will be described in the following sections.

Immunocytochemistry of the nuclear domains

The coordination of all cell functions occurs in the command center located at the nucleus; essential to this control is the ability to regulate the transit of molecular species into and out of the nucleus. The communication between nucleus and cytoplasm that involves the transport of macromolecular complexes is a key step in gene expression regulation (Dreyfuss and Struhl, 1999). This traffic is regulated by very large and complex structures (30-times the size of a ribosome) called nuclear pore complexes (NPCs) that perforate as gateways the outer and inner membranes of the nuclear envelope (NE). Within the nucleus, the structural organization is less defined

than in the cytoplasm. Cytoplasmic organelles are discrete, generally membrane-bound structures, which can be isolated in a considerably pure form to be analyzed and utilized for *in vitro* functional assay. The nucleus is far denser than the cytoplasm, due to the presence of highly polymeric molecules such as DNA and RNAs, and contains a variety of subnuclear structures which lack membranous boundaries. Very complex functions, like transcription, occur at large macro-molecular assemblies, involving some tenths of components that must interact at the right time and place (Stein et al., 2000). This peculiar arrangement of the nucleus and the complexity of the functions occurring in the crowded nuclear space can be studied by a combination of molecular and morphological approaches.

In recent years the cytochemical techniques have gained formidable insights into the functional organization of the nucleus utilizing complementary approaches based on confocal laser scanning microscopy (CLSM) and electron microscopy (EM). CLSM provides the three-dimensional information required to analyze the spatially arranged nuclear domains (Maraldi et al., 1999a). A drawback of fluorescent probe detection within the nucleus is the degradation of nuclear organization caused by the permeabilization procedure that allows the probe to reach the nuclear interior; alterations are also caused by denaturation and deproteination processing required to follow DNA replication by BrdU uptake (Visser et al., 2000). The use of green fluorescent protein (GFP)-labeled probes expanded the capability of CLSM to analyze the nuclear domain activity in vivo. A new chapter concerns the dynamics of some nuclear functions by the use of fluorescence recovery after photobleaching (FRAP) or fluorescence loss in photobleaching (FLIP) techniques to follow the very rapid movements of (GFP)-labeled proteins within the nuclear domains (Phair and Misteli, 2000). Electron microscopy immunocytochemistry attains a spatial resolution of about 10-20 nm with respect to that of 250-700 nm of CLSM methods and a better preservation of nuclear organization, although three-dimensional information can be obtained only by complex methods of reconstruction of serial sections. In several cases, in which the actual function of putative nuclear domains is still debated, such as the chromosome territories (Visser et al., 2000) or the inositol lipid signal transduction systems (Maraldi et al., 1999b), the comparison between CLSM and EM labeling patterns is mandatory.

Organization of nuclear compartmentalization

Development in instrumentation and reagents greatly improved the detection of nucleic acids and proteins by in situ hybridization and immunocytochemical analyses. The combination of non-isotopic methods with computer-aided imaging provided much more complete insights into the actual distribution of genes and regulatory factors that constitute the *in situ* mapping of gene expression.

The role of nuclear domains in the control of developmental expression of cell-growth and tissue-specific genes is of fundamental importance also to understand the molecular basis of pathological alterations (Maraldi et al., 1998). However, the control of gene expression and the identification of the structural organization of the nucleus have been longer considered as minimally integrated questions (Misteli, 2000). This integration has been recognized in other cases; thus there is longstanding acceptance that the nucleolus is the domain at which ribosome biosynthesis occurs (Olson et al., 2000). The import of gene regulatory factors and the export of transcripts have been mapped at the level of nuclear pore complexes (Wente, 2000). DNA replication occurs at distinct foci that associate into larger complexes and early and late replicating chromatin territories correspond to R and G bands of metaphase chromosomes (Zink et al., 1999). Less defined are the structural bases of the functions that control gene expression. This is obviously due to the intrinsic complexity of the phenomenon which is not restricted in time as DNA replication, or in space, as nuclear import/export processes. Furthermore gene expression modulation through signaling molecules and transcription factors (TFs) is an impressively complex event that involves chromatin remodeling, targeting of TFs to regulatory chromatin sequences, recruitment of RNA polymerases, polyadenylation of primary transcripts, recruitment of splicing factors, splicing of hnRNAs, maturation and transport of mRNAs to the nuclear pore complex. All these steps occur in a limited space corresponding to the interchromatin area between the chromosome territory containing the transcribed gene and the nearest nuclear pore (Visser et al., 2000). In this interchromatin domain several macromolecular aggregates have been structurally identified and more or less stringent association with specific functions described. The list of these nuclear components is wide, although probably not exaustive. Coiled (Cajal) bodies (CBs), gemini of coiled bodies (Gems), inter-

chromatin granule clusters (IGCs) or *speckles*, and promyelocytic leukemia (PML) bodies, are quite large structures detectable by fluorescence microscopy as distinct foci changing in number and size during the cell cycle (Lamond and Earnshaw, 1998). Perichromatin fibrils, perichromatin granules, and isolated groups of interchromatin granules are very numerous small structures detectable at the electron microscope level (Stein et al., 2000). An impressive amount of molecular components have been identified by cytochemical methods at these subnuclear structures; most of them are constituents of the transcription machinery or regulatory factors that modulate transcript synthesis, processing and targeting (Mintz et al., 1999; Lewis and Tollervey, 2000).

The existence of nuclear domains, though universally accepted, does not imply that the mechanisms that coordinate the spatial organization of genes, transcripts and regulatory proteins within the nucleus are recognized as well. In fact, the compartmentalization of regulatory factors that sustain a cognate nuclear function could be either maintained by an underlying macromolecular framework, or driven by diffusion mechanisms coupled with transient associations among functionally related components (Shopland and Lawrence, 2000).

These opposite views of nuclear compartment organization and origin are based on a large and compelling amount of experimental data which are substantially non contradictory. However, these results are differently interpreted on the basis of the accepted or refused evidence of a non-chromatin, insoluble nuclear matrix within the nucleus (Fey et al., 1991). DNA replication, ribosome assembly, transcript synthesis and processing are spatially and temporally regulated. The main mechanism through which this occurs is a specific distribution of the factors involved in each of these processes at given nuclear sites. The localization of factors into macromolecular assemblies, morphologically detectable by immunocytochemical methods, is not determined by membrane-delimited territories but by less defined domains to which specific functions are dynamically associated. The question that arises is: which is the basis of nuclear compartmentalization? An obvious reply should be: as in the cytoplasm, a skeletal array of auto-assembling filaments could both maintain and dynamically modify the nuclear domains. As many obvious think, this interpretation is still questioned. Alternative hypotheses on the nucleoplasm organization imply: i) a system which macromolecular complexes are transiently associated with, at given functional sites, mainly through specific interactions with the nuclear matrix components (Kruhlak et al., 2000); ii) a nucleopasmic space in which components diffuse freely but concentrate at site of function through reciprocal interactions (Misteli, 2000).

The nuclear matrix in nuclear domain organization in normal and pathological conditions

The experimental evidence of the structural organization and functional properties of the nuclear matrix has been continuously accumulating in the past twenty years. The nuclear matrix is constituted by different components, the nuclear lamina, strictly associated to the nuclear envelope, the nucleolar remnant, and the inner nuclear matrix, that interacts with the chromosome territories, the interchromatin domains and the nucleolus. Some classes of nuclear matrix proteins have been isolated and the genes encoding them cloned (Nickerson, 2001). The major proteins of the nuclear lamina are lamins B and A/C (Moir et al., 1995; Stuurman et al., 1998; Gruenbaum et al., 2000). Lamins B1 and 2 have been reported to interact with cytoskeletal intermediate filaments (Djabali et al., 1991). Lamins A/C have been also localized at the nuclear interior, conceivably interacting with inner nuclear matrix proteins (Moir and Spann, 2001). The most characteristic proteins of the inner nuclear matrix are the nuclear matrins (Belgrader et al., 1991), the 240-kDa NuMA (He et al., 1995), and the 170-kDa DNA Topoisomerase II a (Berezney et al., 1995; Zini et al., 1994). Another nuclear matrix-associated group of proteins is represented by nuclear actin and by several actin-related proteins (Amankwah and Boni, 1994; Rando et al., 2000).

A class of nuclear matrix proteins specifically bind DNA sequence landmarks, defined S/MARs (scaffold/matrix-associated regions), thus defining the base of topologically constrained chromatin loops, that either insulate genes from the influence of cis-acting elements, or play transcription enhancing activity (Boulikas, 1995). S/MARs as well as some classes of RNPs involved in RNA splicing and transport are so specifically bound to nuclear matrix proteins that can not be removed by high-salt extraction but only by apoptotic proteolytic cleavage (Martelli et al., 1997). A variety of nuclear functions have been demonstrated to require interactions or local-

ization at the nuclear matrix, including gene expression regulation (Nickerson, 2001). Transcription factors are not simply translocated to the nucleoplasm by the presence of nuclear localization signals (NLS). Transcription factor transactivation activity depends on their targeting to the nuclear matrix and, in some instances, to specific nuclear domains, through specific localization signals (Stein et al., 2000). Gene expression and chromatin arrangement are closely associated events being the first dependent on the second. Chromatin arrangement is actively modulated in response to physiological factors by macromolecular assemblies of nuclear matrix-associated proteins, the chromatin remodeling complexes (CRCs), that allow histone-modifying enzymes and transcription factors to reach their targets (Hagmann, 1999; Berger, 2000).

The evidence of a functional relationship between nuclear matrix-associated structures and gene expression is consistent with the finding that a modified subnuclear organization of genes and regulatory factors occurs in pathological conditions like cancer and neuropathies. In both cases there are modifications in the components of the nuclear architecture involved in the control of gene expression. As examples, in normal cells PML transcription factor resides in discrete PML bodies associated with the nuclear matrix, while in promyelocytic leukemic cells the PML protein is genetically rearranged and dispersed throughout the nucleus (Dyck et al., 1994). The subnuclear distribution of ataxin-1 is altered in spinocerebellar ataxia type 1. Because ataxin-1 is nuclear matrix-associated, the pathogenesis of spinocerebellar ataxia involves the progressive disruption of the nuclear matrix and a rearrangement of the nuclear domains (Skinner et al., 1997; Tait et al., 1998). It is therefore evident that the fidelity of the subnuclear organization of factors is essential for integration of the signals that regulate expression of genes that control cell growth and phenotype (Stein et al., 2000).

Emery-Dreifuss muscular dystrophy and other nuclear envelopathies

Emery-Dreifuss muscular dystrophy (EDMD), limb-girdle muscular dystrophy type 1B, hypertrophic cardiomyopathy (Nagano and Arahata, 2000), Dunnigan-type familial partial lipodystrophy (Cao and Hegele, 2000), and axonal neuropathy Charcot-Marie-Tooth disorder type 2 (DeSandre-Giovannoli et al., 2002), have been demonstrated to depend on mutations of nuclear envelope-associated proteins. This fact appears to be particularly significant in confirming the essential link between nuclear organization and the pathophysiology of a group of hereditary diseases identified as nuclear envelopathies (Hegele, 2000).

EDMD is a rare form of X-linked muscular dystrophy, displaying variable phenotypical expressivity, characterized by muscle wasting and weakness, with tendon contractures in the lower leg and the upper arm, as well as by progressive atrio-ventricular conduction defects, that was firstly described by Emery and Dreifuss (Emery, 1989). This recessive muscular dystrophy form (XL-EDMD) is caused by loss of expression of emerin (Bione et al., 1994), a nuclear membrane protein (Nagano et al., 1996; Manilal et al., 1996). An autosomal dominant form of EDMD has been then characterized, demonstrating that this form of the disease (AD-EDMD) is caused by mutations in the gene that encodes alternatively spliced lamins A and C (Bonne et al 1999). Interestingly, the two forms of EDMD show very similar clinical features. Muscle fiber necrosis is rare, while a certain amount of fibers show an high size variability and internal nuclei. Moreover, some muscles show peculiar early contractures, or permanent shortening. At the heart level, cardiomyocyte necrosis and cardiac conduction system degeneration are not usually reported (Emery, 2000). In XL- and AD-EDMD, ultrastructural alterations have been described affecting both chromatin and the nuclear envelope-associated structures, such as the nuclear lamina and the nuclear pores (Ognibene et al., 1999; Sullivan et al., 1999). Heterochromatin distribution, focal absence of heterochromatin, loss of contact between heterochromatin and the nuclear lamina, nuclear lamina thickening, non uniform nuclear pore distribution, are typical ultrastructural changes that characterize the affected phenotype (Figures 1 and 2). In which way emerin and lamin A/C altered expression could result in chromatin arrangement modification, and, in turn, in skeletal and cardiac muscle functional impairment?

The nuclear envelope and the nuclear lamina proteins

The finding that a muscular dystrophy, the EDMD, arises due to defects in nuclear envelope-associated proteins instead of cytoskeletal/plasma membrane-associated proteins, such as in Duchenne muscular dystrophy, was completely unexpected. Therefore, the

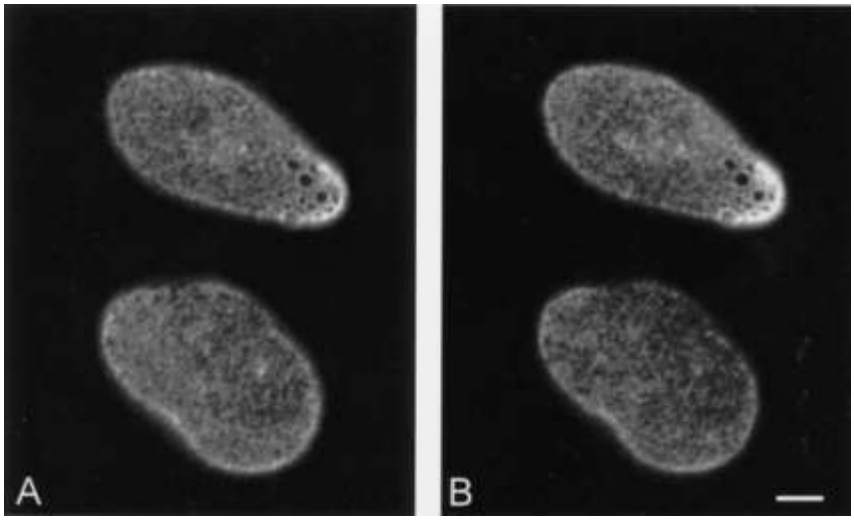

Figure 1. Skin cultured cells from AD-EDMD patient, fluorescence microscopy. A) Anti-lamin A antibody; in the field two nuclei are visible, the bottom one showing an uniform distribution of the labeling all along the nuclear surface, the top one showing local absence of labeling at irregularly shaped black areas; B) Anti-emerin antibody; the labeling pattern is almost identical to that of lamin A, indicating that the altered expression of lamin A/C prevents a regular arrangement of the nuclear envelope-associated protein emerin. Scale bar = 1 µm.

attention has been focused on the nuclear envelope, which represents the site at which both emerin, the protein altered in the X-linked recessive form of EDMD, and lamin A/C, the protein linked to the autosomal dominant form of EDMD, interact. The two proteins are not analogous; in fact, emerin is a transmembrane protein associated to the inner nuclear membrane, whilst lamin A/C is a member of the intermediate filament family located at the nuclear lamina. However, the two proteins interact, since emerin is retained at the inner nuclear membrane owing to direct or mediated interactions with lamin A/C (Fairley et al., 1999). Emerin is a member of the lamina-associated nuclear envelope (NE) membrane proteins (Tews, 1999); the other known members being: lamin B receptor (LBR), lamina-associated polypeptide 1 (LAP1 A, B, C), and LAP2β, MAN1, and nurim (Morris and Manilal, 1999). All these proteins have hydrophobic domains that constitute putative transmembrane anchoring sites, and some of them have been reported to present lamin- and chromatin-binding sequences (Cartegni et al., 1997; Manilal et al., 1999; Östlund et al., 1999). These molecular interactions con-

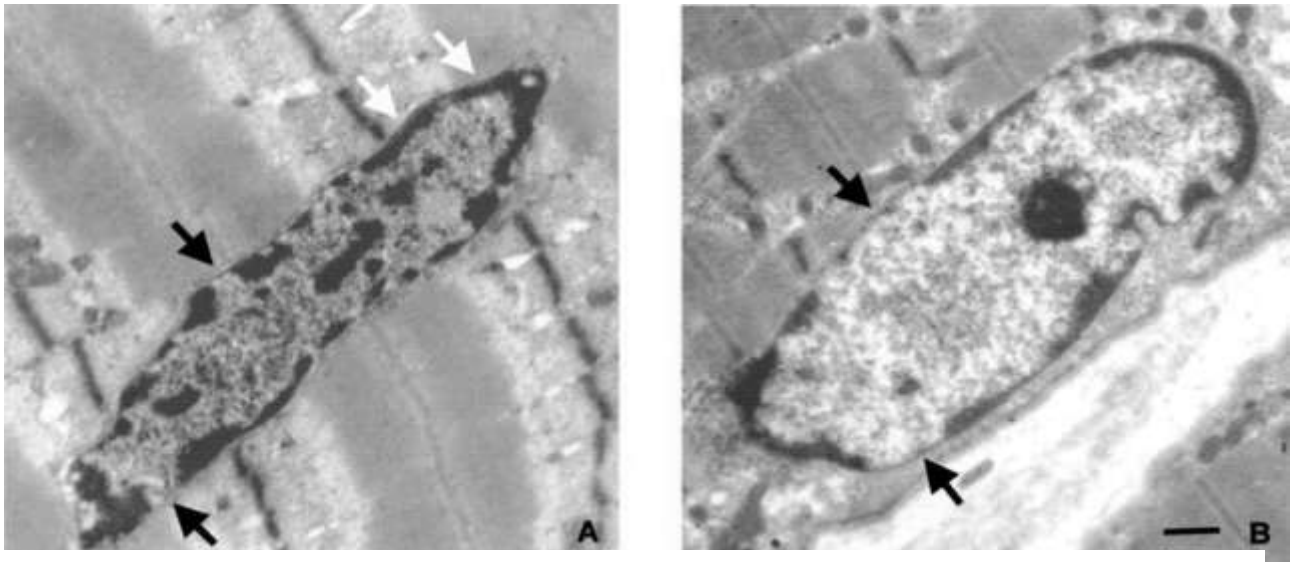

Figure 2. Skeletal muscle biopsies, electron microscopy. A) XL-EDMD; B) AD-EDMD. Nuclei show typical ultrastructural alterations with local absence of peripheral heterochromatin (black arrows) and thickening of the nuclear lamina (white arrows). Scale bar = 0.5 µm.

tribute to the chromatin arrangement (Figure 3).

Emerin is a type II integral membrane serine-rich protein; it is linked to the inner nuclear membrane by its hydrophobic C-terminal tail. This typical localization depends on interactions with other nuclear membrane proteins as well as on interactions with nuclear lamina or nuclear matrix proteins (Wilson, 2000). Transmembrane sequence is involved in emerin localization throughout the ER system, whilst at least two separate sequences are essential for nuclear envelope targeting; interestingly, emerin, when overexpressed, can reach also extra-nuclear membrane districts (Östlund et al., 1999). The identification of emerin also at cytoplasmic sites has been reported (Cartegni et al., 1997; Östlund et al., 1999; Squarzoni et al., 2000), although its localization at the intercalated discs in the heart (Cartegni et al., 1997) has been partly disregarded by the evidence of nuclear membrane exclusive staining by means of monoclonal antibodies (Manilal et al., 1999). Emerin can be considered a bridge between the inner nuclear membrane and the nuclear lamina, since it remains strongly associated with the nuclear lamina in purified nuclear matrix preparations (Squarzoni et al., 1998). Diffusion of emerin is however limited by interactions with proteins belonging to nuclear structures that face the inner nuclear membrane, that is the nuclear lamina and the peripheral heterochromatin. Lamins A and C interact with emerin as demonstrated by co-immunoprecipitation and blot overlay experiments (Fairley et al., 1999; Sakaki et al., 2001), and by knock-out mice model (Sullivan et al., 1999). A putative emerin partner is the chromatin protein BAF (Barrier to Autointegration Factor), that has been demonstrated to bind LAP2 (Furukawa et al., 1998). It has been suggested that BAF-binding proteins might influence chromatin arrangement and expression (Wilson, 2000).

Lamins are differentially expressed during development and differentiation; in man, lamins B1 or B2 are expressed in almost all cell types, while lamins A and C are expressed primarily in differentiated non-proliferating cells, suggesting that they are involved in gene expression. The LMNA gene products, lamins A and C, are produced by an alternative splicing at exon 10 of LMNA, so that the two proteins share the first 566 residues, but have distinctive C termini (Stuurman et al., 1998). Lamin A but not lamin C can be modified by farnesylation. LMNA mutations, by affecting charge or hydrophobicity, could destabilize the α-helix interactions at the rod domain, preventing dimerization (Hutchison et al., 2001; Genshel and Schmidt, 2000). Because of differential expression of lamins and lamin-associated proteins in different tissues, the LMNA mutations can differently affect the tissue pathophysiology. Moreover, lamin A/C could also interact with TFs such as SREBP-1, affecting gene expression (Wilson, 2000). Finally, abnormal interaction of mutated lamins with cytoskeletal or sarcomeric proteins (belonging to the intermediate filament family) might occur in the skeletal and cardiac cells in EDMD and hypertrophic cardiomyopathy 1A (Herrmann and Aebi, 2000). Lamins are type V intermediate filament proteins that form orthogonal rather than linear arrays. The complexity of the nuclear lamina ultrastructure, including both lamins and lamin-binding proteins, appears to be strictly related with the evolutional complexity in eukaryotes (Cohen et al., 2001). On the other hand, the evolution of nuclear lamina increases the efficiency of nuclear envelope disassembly, which is a prerequisite of a more efficient mitotic process and which provides new mechanisms to modulate chromatin expression through exposure to cytoplasmic factors. Furthermore, a more sophisticated nuclear lamina probably confers selective advantages, possibly due to improved chromatin organization, nuclear signaling and gene expression (Gotzmann and Foisner, 1999). This facts should be taken into account to determine how mutations in nuclear lamina proteins could cause inherited diseases. Nuclear lamina components can affect other cell responses, some of which could be involved in EDMD pathophysiology, such as apoptosis, chromatin remodeling and transcriptional regulation.

Hypotheses on the pathophysyology of EDMD

Some hypotheses have been advanced in order to explain the role of mutant nuclear envelope-associated proteins in the pathophysiology of EDMD (Figure 3). The first is that of the mechanical stress, analogous to that proposed in other muscular dystrophies (Manilal et al., 1999). The mechanical stress has been suggested to cause, in muscular dystrophies involving cytoskeletal/plasma membrane-associated proteins, a progressive damage of the myofibrils, due to a not coordinated association between the extracellular matrix and the cytoskeleton during contraction. A similar situation is less plausible in EDMD; however, emerin may be part of

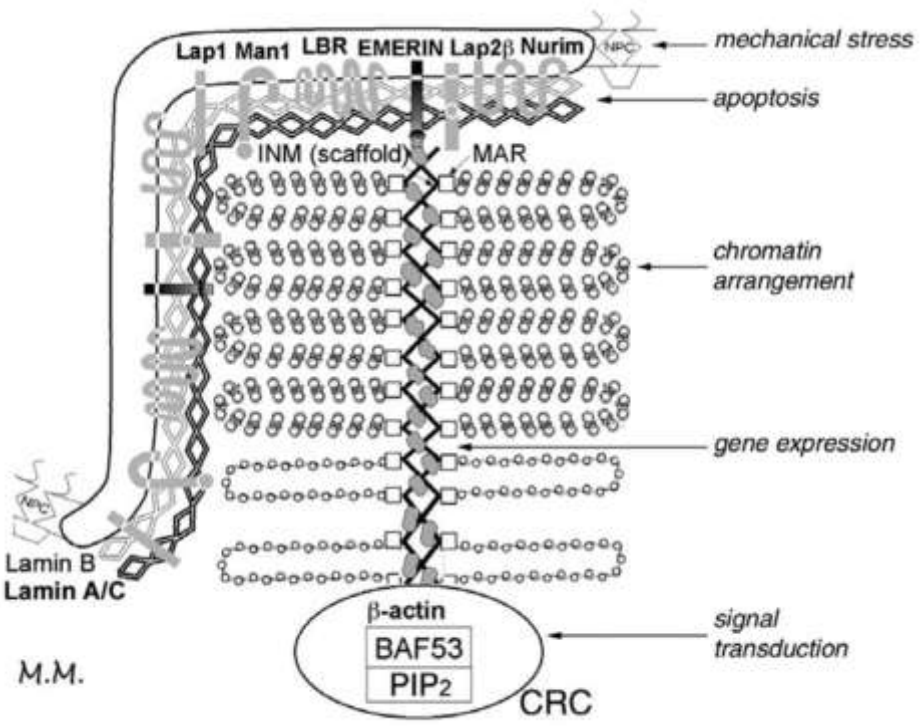

Figure 3. Molecular interactions among nuclear envelope-associated proteins, nuclear lamina, nuclear matrix and chromatin. INM (scaffold): only Lamin A and β-actin are represented; CRC: PI(4,5)P_2-dependent Chromatin Remodeling Complex BAF. Different mechanisms possibly involved in EDMD pathophysiology are indicated as affecting specific nuclear targets (arrows). Loss of emerin or altered expression of lamin A/C could result in: damage at the nuclear envelope membranes (mechanical stress); unstable nuclear lamin (apoptosis); altered nuclear matrix/chromatin interactions affecting gene silencing by heterochromatization (chromatin arrangement); altered binding to the nuclear lamina/matrix of trancriptional regulators (gene expression); altered mechanisms of CRC involving PI(4,5)P_2-dependent actin polymerization that affect nuclear matrix/chromatin interactions (signal transduction).

a nucleo-cytoskeletal network which protects the nucleus of contractile cells from the mechanical stress (Maniotis et al., 1997). Accordingly, mutations of lamin A/C could determine an increased sensitivity to the stress in cardiac and skeletal muscle nuclei which lack lamin B1 (Manilal et al., 1999). Nuclear fragility as a cause of the defects occurring in EDMD has been suggested in knockout mice lacking lamin A/C by the presence of muscle wasting and contractures not at birth, but after some weeks (Sullivan et al., 1999). These symptoms are accompanied by an altered nuclear shape, due to the absence of lamin A/C and abnormal distribution of emerin (emerin is mislocalized to the ER, suggesting a lamin A/C-emerin interaction at the NE), that occurs not only in muscle cells, but also in other tissues (Sullivan et al., 1999). Therefore, the nuclei of all tissues, once isolated, are fragile, but conceivably only muscle fibers, due to the mechanical stress, undergo nuclear alterations. In skeletal muscle syncytium the damage is limited, because not all nuclei are damaged simultaneously. In contrast the damage of cardiomyocytes will be cumulative and could lead to conduction blocks (Hutchison et al., 2001).

Another hypothesis has been recently advanced, that takes into account the fact that muscular dystrophies are degenerative diseases that involve two recognized mechanisms for cell death, apoptosis and necrosis, that might be not mutually exclusive. In EDMD, that seems not to involve inflammatory responses, the altered expression of nuclear envelope-associated proteins could have no deleterious effect on most cells, but it might make cardiac and skeletal muscle cells more susceptible to programmed cell death (Morris, 2000). However, a direct demonstration is still lacking, since apoptosis

is rather difficult to identify in adult tissues, because it has to be identified as an ongoing process affecting a small proportion of cells, that maintain cycling properties, such as satellite cells.

A further hypothesis, which is progressively more accepted, suggests that emerin and lamin A/C may regulate gene expression by interacting with specific transcription factors or DNA sequences (Östlund et al., 1999; Tsuchiya et al., 1999), as well as modulating chromatin arrangement (Wilson, 2000). This hypothesis is sustained by several experimental findings: a cell cycle-dependent binding of LAP2β to lamin B1 controls the increase of nuclear volume which occurs in cycling cells and allows the activation of genes involved in the triggering of S phase (Gruenbaum et al., 2000). Emerin too has a role in cell-cycle dependent events, being phosphorylated in different forms which are expressed in a cell cycle-dependent way (Manilal et al., 1999). Lamins are capable of interacting with Rb, an oncosuppressor gene product that regulates cell cycle progression by recruiting histone deacetylase complexes that cause chromatin condensation (Mancini et al., 1994). Finally, in XL-EDMD patients (Ognibene et al., 1999) and in lamin-A/C-null mice (Sullivan et al., 1999), the peripheral heterochromatin layer underlying the NE is altered, suggesting that the chromatin attachment to the NE is an essential mechanism for the regulation of the cell cycle and of gene expression (Wilson, 2000). The modulation of chromatin arrangement that might influence gene expression should occur both at the nuclear envelope-associated chromatin as well as at other intranuclear sites, where lamin A has been localized (Neri et al., 1999; Hutchison et al., 2001).

An alternative hypothesis takes into account the influence that NE-associated proteins can exert on signal transduction. On the other hand, also the pathogenesis of muscular dystrophies involving cytoskeletal/cell membrane-associated proteins could be explained by altered signal transduction mechanisms (Rando, 2001). In fact, an altered localization of filamin 2 (a sarcoglycan interacting protein), occurs in limb-girdle and Duchenne MD; this protein is involved in cytoskeletal actin reorganization and signal transduction cascades associated with cell differentiation in a way that recalls α 7 integrin subunit (Thomson et al., 2000), calpains and caveolins (Razani et al., 2000). The dystrophin-glycoprotein complex, originally believed to play a structural role, is more likely considered to dynamically modulate signal transduction cascades affecting cell development and the acquisition and maintenance of a differentiate phenotype (Yoshida et al., 2000; Chen et al., 2000). NE-associated proteins might affect gene expression and cell differentiation being part of structural platforms for signaling systems (Ellis et al., 1998), located at the NE or within the nucleus. The signaling hypothesis partly overlaps the gene expression hypothesis, suggesting a way by which NE-associated proteins can interfere not only with chromatin arrangement but also with the release of signaling molecules that modulate transcriptional regulators.

Signal transduction at the nuclear level and the modulation of chromatin arrangement

The nucleus represents not only the final target of signaling molecules but also an autonomous cellular compartment where signaling molecules are generated. In fact, one of the main class of signaling molecules, the phosphoinositides that give rise to the second messengers diacylglycerol and IP3, or act directly as second messengers such as $PI(4,5)P_2$, is not only present at the cell membrane (where the receptors of several hormones and growth factors are located), but also at the cell nucleus (Maraldi et al., 1999b). The inositol lipid signaling system at the nucleus is characterized by the presence of: i) specific isoforms of the lipid kinases that phosphorylate the inositol ring, ii) phospholipases that hydrolyze polyphosphoinositides, iii) protein kinases that are the targets of the lipid-derived second messengers (Irvine, 2000; D'Santos et al., 1998). The nuclear signaling system, moreover, responds in a specific manner to agonists that modulate cell proliferation or differentiation (Cocco et al., 2001; Chi and Crabtree, 2000). Interestingly, the responses induced by these effectors are not a mere duplication of those occurring elsewhere in the cell (Irvine, 2000). Some elements of the system are located at the nuclear envelope, other at intranuclear domains involved in RNA splicing, such as interchromatin granules that are linked to the nuclear matrix (Maraldi et al., 1999b). It is conceivable that alteration in the expression of NE-associated proteins can interfere with signal transduction at the nuclear level.

One of the most impressive phenomena that follows cell activation (for example, peripheral lymphocyte exposure to antigens) is the massive chromatin rearrangement that occurs in few minutes and pre-

cedes gene activation. The phenomenon consists in the decondensation of a large amount of heterochomatin, causing the enlargement of the cell volume, and is mediated by multimeric protein complexes, the chromatin remodeling complexes (CRCs) (Wang et al., 1996). Chromatin decondensation is therefore a prerequisite that allows transcription factors to activate specific gene promoters. Chromatin organization is thought to be dependent on the relationships between nucleohistone fibers and the nuclear matrix (Nickerson, 2001); chromosome domains that persist into interphase are structurally connected with the inner nuclear matrix and with the nuclear lamina. It is therefore conceivable that alteration in the expression of lamins and lamin-associated proteins can interfere with chromatin arrangement (Boyle et al., 2001). Heterochromatin dynamics appears to be related to the cell cycle. In late S, in fact, HC is released from the NE and moves towards the replication sites and then returns to the envelope and could be regulated by lamins and LAP2, which are phosphorylated at specific sites (Dreger et al., 1999). Furthermore each cell type has a characteristic nuclear shape and a typical HC pattern, which conceivably affects its transcriptional expression. Both these phenotypic characters are acquired by descending cell lineages.

The molecular basis of chromatin remodeling through a nuclear localized signal transduction system, has been recently identified. Several chromatin remodeling complexes (CRCs) have been characterized in yeast, Drosophila, and mammals (Wang et al., 1996; 1998). All of the CRCs appear similar in their ability to modify nucleosomal structure and allow binding of transcription factors. In higher eukaryotes CRCs are multisubunit 2 MDa protein complexes, among which the complexes called Brahma-related gene associated factors (BAF) have been extensively studied (Wang et al., 1996). In human lymphocytes, the BAF complex, constituted by BRG1, β-actin and the actin-related protein BAF53, is actively induced to associate to the nuclear matrix/chromatin within 10 min after antigen receptor stimulation, before detectable increase in transcription or protein synthesis (Zhao et al., 1998). The activation of the BAF complex, leading to an impressive and sudden chromatin decondensation, is triggered by PIP2 levels that control the association of the complex with the nuclear matrix. In turn, PIP2 modulates actin polymerization in the BAF complex by displacing nuclear actin-binding proteins, suggesting a direct interface between chromatin regulation and signal transduction at the nuclear level (Maraldi et al., 1999b; Zhao et al., 1998).

These experimental data support the following conclusions: i) chromatin remodeling is a rapid and efficient mechanism that constitutes a prerequisite of gene activation (Kadonaga, 1998; Kingston et al., 1996); ii) CRCs are nuclear matrix-associated systems capable of responding to signaling molecules to rapidly modify the nuclear arrangement through actin polymerization (Zhao et al., 1998); iii) inositol lipid-derived second messengers, capable of interfering with actin-binding proteins, constitute a signaling system present within the nucleus and responding to pathophysyological stimuli (Maraldi et al., 1999b).

It is conceivable that mutations in lamins A/C and emerin can affect gene expression through a signaling mechanism capable of modulating chromatin arrangement in response to external stimuli. Emerin-deficient nuclei, or lamin A/C-mutated nuclei might present subtle defects arising from altered chromatin structure that not necessarily affect gene expression in all cell types (Wilson, 2000). Some cell types, in fact, like lymphocytes, alternate long quiescent phases with sudden activation periods; such changes require a profound chromatin remodeling and dramatic reprogramming of the whole nuclear size and shape (Zhao et al., 1998). Mesenchymal cells arising in the bone marrow differentiate into the various cells showing alterations in patients affected by envelopathies. Adipocytes, that are affected in the Dunnigam-type familial partial lipodystrophy (Cao and Hegele, 2000), and motor neurons, altered in CMTD Type 2 (DeSandre-Giovannoli et al., 2002), do not derive from the mesenchyma (Procop, 1997) but, as muscle cells, are usually non proliferating. Subtle alteration in chromatin arrangement affecting gene expression in given situations might negatively affect mainly long-lasting cells.

Nuclear envelope/chromatin interactions in normal conditions and in EDMD

Proteins of the inner nuclear membrane, nuclear lamina and chromatin are highly interacting (Figure 3). Emerin, LAP2β and MAN1 are characterized by the LEM box, constituted by a 43-residue motif by which these proteins can interact with DNA-binding proteins capable of affecting chromatin arrange-

ment (Furukawa et al., 1998; Lin et al., 2000). Other members of the nuclear envelope/nuclear matrix system interact with DNA-binding proteins, such as LBR with the heterochromatin protein HP1 (Ye and Warman, 1996), and lamins with histones (Goldberg et al., 1999) and the retinoblastoma (Rb) protein (Brehm et al., 1998). Particularly interesting is the case of Rb, which inhibits S-phase related genes by recruiting histone deacetylase that represses transcription affecting chromatin condensation (Brehm et al., 1998). Emerin, and lamin A/C can bind actin (Farley, 1999, Sasseville and Langelier, 1998), a nuclear matrix-associated protein. Other interactions have been recently reported between HA95, a protein tightly associated with chromatin and the nuclear matrix, and a complex constituted by LBR, LAP2β and emerin (Martin et al., 2000). Thus, the chromatin provides multiple anchoring site for the nuclear envelope, so that the nuclear envelope-associated proteins can modulate the chromatin arrangement.

The mechanism of chromatin remodeling by CRC involves a PIP2-dependent modulation of the association of the complex to the nuclear matrix through the PIP2-responsive actin-regulatory protein BAF53 (Zhao et al., 1998). Chromatin remodeling is the prerequisite to trigger the release of chromatin template restriction, allowing specific transcription factors to bind chromatin itself (Lewin, 1994). A specific $PI(4,5)P_2$-binding, actin-regulatory protein, the nuclear CapG, is preferentially phosphorylated compared with cytosolic CapG (Yu et al., 1990). Since phosphorylated CapG, in concert with Ca^{++}, can compete with nuclear PI 3-kinase for its $PI(4,5)P_2$ substrate, CRC could be modulated in a multiple way by both PLC-β1 and PI 3-kinase (Maraldi et al., 2000). The interaction among emerin, lamin A/C and nuclear actin can affect the chromatin arrangement that precedes transcription factor-modulated transcriptional activity. The sudden transition from an inactive state to an activated state, controlled by the nuclear signaling effector $PI(4,5)P_2$, causes the association of BAF to the inner nuclear matrix (INM) scaffold. As a consequence, the solenoid fibers attached to the scaffold by MAR sequences loose their compact arrangement and become accessible to histone-modifying enzymes, TFs and RNA polymerase. In fact, many of the enzymes and complexes required for chromatin remodeling and gene activation are present at nuclear matrix-associated sites, which also contain the MARs of the chromatin loops. The inner nuclear matrix 10 nm filaments show a non-helical arrangement and, like intermediate filaments, have an axial repeat of 23 nm. They should include hnRNP core proteins A2/B1, NuMA, lamins, nuclear actin and actin-binding proteins that can bind NuMA (Nickerson, 2001). As suggestively stated in a review on the nuclear assembly (Gant and Wilson, 1997), chromatin structure may be the *sleeping giant* of nuclear matrix structure. The decondensation of mitotically condensed chromatin is thus assumed to be important for, or at least linked to, proper nuclear envelope growth (Gant and Wilson, 1997). It is reasonable that also a proper nuclear envelope organization may be essential to the mechanism of controlled chromatin decondensation and gene expression.

Alterations in either emerin or lamin A/C might impair the relationships between the nuclear envelope and the nuclear matrix-associated actin, thus affecting the CRC-controlled chromatin decondensation (Figure 3). It has been reported in EDMD and in lamin A-deficient mice that peripheral condensed chromatin is altered or absent. Thus it has been hypothesized that a failure to correctly sequester transcriptionally inert chromatin at the nuclear periphery might contribute to the pathology of EDMD by perturbing gene expression. This interpretation is probably misleading, since it implies that nuclear envelope-associated proteins are involved in anchoring gene-poor chromosomes at the nuclear periphery. This hypothesis, indeed, has been partly contradicted by the finding that in cells lacking emerin, the heterochromatin of chromosome 18 is normally localized (Boyle et al., 2001). A more correct interpretation takes into account that emerin and lamin A/C are not exclusively localized at the nuclear rim, but, in particular moments of the cell cycle, also within the nucleus (Morris and Manilal, 1999; Hutchison et al., 2001; Maraldi et al., 2002). At these multiple sites they might interact with actin at the CRCs which can modulate chromatin arrangement throughout the nuclear domains. This implies that the nuclear matrix supports the structural properties of the nucleus and accounts for modifications in gene expression associated with differentiation necessary to sustain phenotypic requirements in specialized cells. Therefore, an altered chromatin remodeling could be particularly effective during the first phases of cell differentiation, being the sudden chromatin decondensation induced by environmental

factors a basic mechanism that precedes more subtle modulations of chromatin arrangements through histone acetylation, methylation and phosphorylation (Berger, 2000) that allow the binding of cell lineage-specific transcription factors.

Involvement of nuclear envelope-associated proteins in myogenic differentiation

In different tissues, the presence or absence of different lamins and/or lamin-associated proteins, could result in different phenotypical effects. Abnormal interactions with cytoskeletal or sarcomeric proteins might be responsible of skeletal and cardiac myocyte abnormalities (Hegele, 2000). Indeed, mAKAP, an A kinase-associated protein, is located exclusively at the inner nuclear matrix of skeletal and cardiac muscle cells, so that PKA localization and function should be altered by a damaged lamina (Hutchison et al., 2001; Kapiloff et al., 1999).

The organization and genesis of skeletal muscle phenotype appears to be unique for the following characteristics: presence of multiple nuclei whose influence on the fiber development may be strictly coordinated by signaling molecules; accumulation of metabolic defects in non-proliferating terminal cells; possibility that the same proteins play different roles in different moments of the complex differentiation pattern of the muscle cells (Yun and Wold, 1996). The fusion of satellite cells to form myotubes involves re-entry into the cell cycle (Schultz, 1996). Both emerin (Lattanzi et al., 2000) and lamin A/C (Lourim and Lin, 1989) have been reported to be involved into the myogenic differentiation. Emerin and lamin A/C absence or altered expression in EDMD might impair the response to cell cycle-mediated events in satellite cells. This would imply that cell cycle-dependent functions of NE-associated proteins are mainly relevant in satellite cells, or that there is a genetic redundancy in other tissues, which is absent from muscle (Ellis et al., 1998). Evidence of the involvement of emerin in myogenic differentiation has been obtained evaluating the expression of the protein in cultured myoblasts (Lattanzi et al., 2000). In fact, emerin is present in cycling myoblasts and, in higher amounts in the myotubes, in which also the expression of MyoD is high, and less represented in resting mononucleated cells, which represent quiescent reserve cells where also MyoD is down-regulated (Yoshida et al., 1998). Moreover, during myogenic differentiation, emerin is expressed also at the cytoplasm, while it is targeted to the NE in developed muscle fibers, suggesting a differential role of the protein in different moments of the muscular phenotype acquisition (Lattanzi et al., 2000). Accordingly, in adult muscle, a deficiency in emerin expression in satellite cells could impair regeneration or mass development of the muscle.

These findings, as well as the restriction of EDMD defects to defined skeletal districts, suggest that interaction with other proteins (transcription factors, chromatin-binding proteins, muscle-specific cytoskeletal proteins) can account for multiple role of emerin in muscle development (Lin et al., 2000). During myogenesis emerin may constitute a network between the cytoplasm and the nucleus allowing the coordination of signal transduction, as hypothesized for lamin B (Georgatos and Blobel, 1987). The organization of the cytoplasmic array of myofilaments is under the control of the intermediate filaments desmin, vimentin, nestin (Capetanaky et al., 1997; Li et al., 1997; Vaittinen et al., 1999) and the correct location of the multiple nuclei may require a close connection between nucleoskeletal and cytoskeletal structures. Once cell differentiation is achieved, emerin is no longer expressed at the cytoplasmic level but targeted exclusively to the NE, at least in skeletal myofibers. The absence of emerin gives rise to apparently normal muscle cells, in which, during the initial steps of myogenesis, its role is partly sustained by other differentiating factors. However, in the adult, the absence of emerin in the satellite cells may result in the impossibility of a physiological regeneration by transformation of satellite cells into myotubes, thus causing a progressive muscle wasting.

In EDMD severe disfunctions occur at the cardiac conduction system that, after the development of arrythmias, can lead to sudden death even in the absence of deep alterations of the contractile cardiac cells. This suggests that NE-associated proteins altered in EDMD do not alter cardiac muscle contraction but affect structures that mediate the conduction pathway. The reported presence of emerin at intercalated discs in normal heart tissue (Cartegni et al., 1997) is suggestive of a possible cytoplasmic role of the protein (Squarzoni et al., 2000). The structure of emerin is consistent with a role of molecular targeting in different membrane districts, being the interaction through the LEM-box capable of recognizing different proteins belonging to structural platforms (Lin et al., 2000). Multi-protein

platforms are considered as the main functional module in signal transduction; protein/protein interactions through SH2 domains, lipid/protein interactions through PH domains are at the basis of signaling systems, whose localization in membrane districts could depend on local domains (lipid microdomains, caveolae) or by intramembrane target proteins. Emerin and lamins may be part of the signaling system, mainly, but not exclusively, located at the nuclear level. Alterations of these proteins in particular districts could be responsible for the different phenotypical alterations that occur in mesenchymal cells in EDMD (skeletal muscle cells, cardiomyocytes), especially during the development. Interestingly, during muscle cell differentiation from myoblast to myotube, lamin A/C amount increases at the nuclear lamina level (Chaly et al., 1996) in correspondence with changes in the peripheral chromatin arrangement.

Conclusions

The identification of inherited distinct diseases that are caused by mutations in lamins and lamin-associated proteins is one of the most intriguing findings not only in the pathophysiology of these diseases but also in that of basic cell biology. In fact, given the precedents of seemingly-unrelated diseases, such as EDMD and Dunnigan lipodystrophy, other nuclear envelopathies are likely to emerge. On the other hand, despite the progress in molecular genetic studies on the nuclear envelope proteins, the mechanism by which mutations in emerin or in lamin A/C selectively cause damages in skeletal and cardiac muscles has not been clarified. This indicates that our knowledge about the role of the nuclear envelope-associated proteins on the functional activities of the nuclear domains is still unsatisfactory. Provided that the mechanical stress hypothesis is not completely convincing, the possibility that gene expression could be affected by mutations in lamin A/C and emerin appears plausible and testable.

Future prospects might take into account some experimental findings that are emerging in recent years: i) gene expression regulation occurs in steps, being the chromatin decondensation a prerequisite with respect to transcription factor binding to gene promoters; ii) chromatin arrangement is modulated by chromatin remodeling complexes, macromolecular aggregates that respond to signals and utilize actin and actin-associated proteins of the nuclear matrix to modify the chromatin loop-nuclear matrix relationships; iii) within the nucleus, located at both the nuclear envelope and at the inner nuclear matrix, an autonomous signaling system, based on polyphosphoinositides, has been demonstrated to affect chromatin arrangement through a direct influence on actin-binding proteins of the chromatin remodeling complexes.

Future investigations will demonstrate whether mutations in lamin A/C and emerin can selectively affect chromatin arrangement and gene expression of the myogenic lineage during differentiation and/or repair.

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