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Subnuclear Dynamics and Transcription Factor Function

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Abstract At a simplistic level, the nucleus can be thought of as singular organelle with a nuclear envelope designed to isolate the biochemical reactions required for gene transcription and DNA replication from the cytoplasm. It has become increasingly clear, however, that many higher levels of organization exist within the nucleus. A functional consequence of this organization is that nuclear processes that include transcription, RNA processing, and DNA synthesis are isolated to specific intranuclear domains to ensure efficiency. With the advent of GFP technologies and increasingly sophisticated instrumentation, we have continued to dissect the relationship between organization and function, in particular using live cells and ligand-dependent steroid receptors as a model system. These new opportunities have provided further insight into receptor function and the dependence upon intranuclear dynamics that take place within minutes of hormone addition. J. Cell. Biochem. Suppl. 35: 99–106, 2000. © 2001 Wiley-Liss, Inc.

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The nucleus is a complex organelle containing subnuclear domains that serve to partition the machineries required for various types of nuclear metabolism. A critical question addressed in this brief overview is the dynamic relationship between nuclear organization, gene expression, and the nuclear matrix. Sites of newly synthesized mRNA as visualized by bromouridine incorporation have a focal distribution within the nucleus. As expected, RNA polymerases show a hyper-speckled distribution and colocalize with newly made RNA [Jackson et al., 1993; Wansink et al., 1993]. In particular the active, hyperphosphorylated large subunit of RNA polymerase II, forms from several hundred to 4,000 foci indicating that mRNA transcription is limited to discrete sites within the nucleus [Jackson et al., 1998; Cook, 1999].

While the basis of the organization of the nuclear domains involved in transcription remains to be determined, growing evidence indicates that the nucleus contains a network of structured fibers connected to the nuclear lamina. This structure, commonly called the nuclear matrix (NM), is defined as the compartment of the nucleus, which is resistant to detergent treatment, DNaseI digestion and high salt extraction [Nickerson, 1995]. Ultrastructural analyses following these treatments reveal the existence of a structural network comprised of highly branched filaments within the nucleus. Based on the assumption that an extraction could result in artefactual "structures" and the inability to identify specific structural proteins, the NM has been the subject of controversy [Pederson, 2000]. However, it is extremely important to point out that alternative approaches that utilize physiological extractions or non-invasive microscopic techniques provide ample evidence that the NM is a bona fide structure [Jackson and Cook, 1988; Nickerson et al., 1997; Hendzel et al., 1999; Wan et al., 1999; see Nickerson, 2001].

Since much of the protein machinery for transcription, RNA splicing and DNA replication is found associated with the NM, this structure may be the basis for their organization [Reviewed in Berezney et al., 1995; Nickerson et al., 1995; Stein et al., 2000; Nickerson, 2001]. While many of the basal transcription components have been found organized in

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NM-bound foci, a question arises as to how transcription factors that regulate transcription during the cell cycle or in response to outside stimuli are organized. In other words, are foci formation and NM interaction fundamental steps in the regulation of the response mediated by transcription factors?

The composition of NM varies in a cell-cycle and differentiation specific way and this may serve to modulate how cells respond to different stimuli [Stein et al., 1996]. An example is represented by the retinoblastoma protein (RB), a prototypical tumor suppressor that interacts with several transcription factors and other proteins [Riley et al., 1994]. RB associates with the NM in a cell-cycle dependent manner (during G1), pointing to the dynamic nature of NM association [Mittnacht and Weinberg, 1991; Mancini et al., 1994]. The importance of this NM association is highlighted by the observation that inactivating RB mutations found in several different tumors result in a complete loss of NM association. Many transcription factors involved in tissue-specific differentiation have also been shown to partition with the NM fraction [van Wijnen et al., 1993]. Specific examples of these are the POU-class transactivators, Pit-1 and Oct-1, which are distributed between soluble and NM-bound fractions in an approximately 3:1 ratio [Kim et al., 1996; Mancini et al., 1999]. The finding that soluble and insoluble pools of transcription factors exist suggests that NM-binding is a regulated process. In the case of Pit-1, several inactivating mutations that lead to dwarfism (e.g., Pit-1 is required for growth hormone synthesis) result in tighter NM association [Mancini et al., 1999]. New live cell approaches utilizing photobleaching indicate inactivating mutations affect Pit-1 mobility in a multi-step fashion, prior to immobilization (see below, Sharp ZD, Stenoien DL, Mancini MG, Mancini MA, manuscript in preparation). Taken together with the RB data, alterations in NM binding dynamics either by decreasing (as with RB mutations) or increasing (Pit-1 mutants) severely affects transcription factor function. Furthermore, mutations that disrupt the dynamic associations between transcriptional regulators and the NM may have functional consequences related to tumor suppression or pituitary development and function.

Steroid receptors associate with the NM in a tissue specific and hormone dependent manner [Barrack, 1987]. The intracellular distribution of steroid receptors in the absence of ligand ranges from being predominantly cytoplasmic in the case of the androgen receptor (AR) to predominantly nuclear in the case of the estrogen receptorα (ER). This suggests that nuclear localization of steroid receptors is not sufficient to ensure activity and subsequent events are required for transcription initiation. With the revolutionary development of functionally active green fluorescent protein (GFP) fusion proteins and high-resolution microscopic techniques, it is possible to detect subtle changes in steroid receptor localization immediately following addition of agonist. In the case of GFP-ER, agonist addition results in redistribution from nuclear diffuse to discrete foci within minutes [Htun et al., 1999; Stenoien et al., 2000], suggesting that it is the focal GFP-ER that is transcriptionally competent. In this vein, some AR antagonists cause nuclear translocation of GFP-AR but fail to form foci [Tyagi et al., 2000; (Simeoni S, Stenoien DL, Mancini MG, Mancini MA, manuscript in preparation)]. Real-time extraction studies to assess the solubility of GFP-ER in individual cells [Stenoien et al., 2000, see below] demonstrate a strong link between foci formation and NM association. Furthermore, GFP-ER deletion analyses also support the idea that reorganization and NM association are related since they require the same molecular domains (Stenoien DL, Mancini MG, Patel K, Smith CL, Mancini MA, manuscript in preparation). ER antagonists also shift ER to the NM and reorganize the receptor although in some cases the antagonist induced reorganization is distinct from that observed with agonist [Stenoien et al., 2000]. These data suggest that binding to the NM may have other functions in addition to the organization of focal sites of transcription factors.

While the agonists and antagonists thus far tested each cause ER to associate with the NM, only agonist-bound ER recruits functionally important coactivator molecules such as steroid receptor coactivator 1 (SRC-1) [Stenoien et al., 2000]. Several motifs with the sequence LXXLL are found in SRC-1 in a region comprising amino acids 570–780. A yellow fluorescent version of this protein (YFP-SRC570-780) is recruited to cyan fluorescent protein-ER (CFP-ER) foci following agonist addition and becomes resistant to detergent extraction (Fig. 1). The YFP-SRC570-780 by itself is predominantly cytoplasmic since it lacks the NLS found at

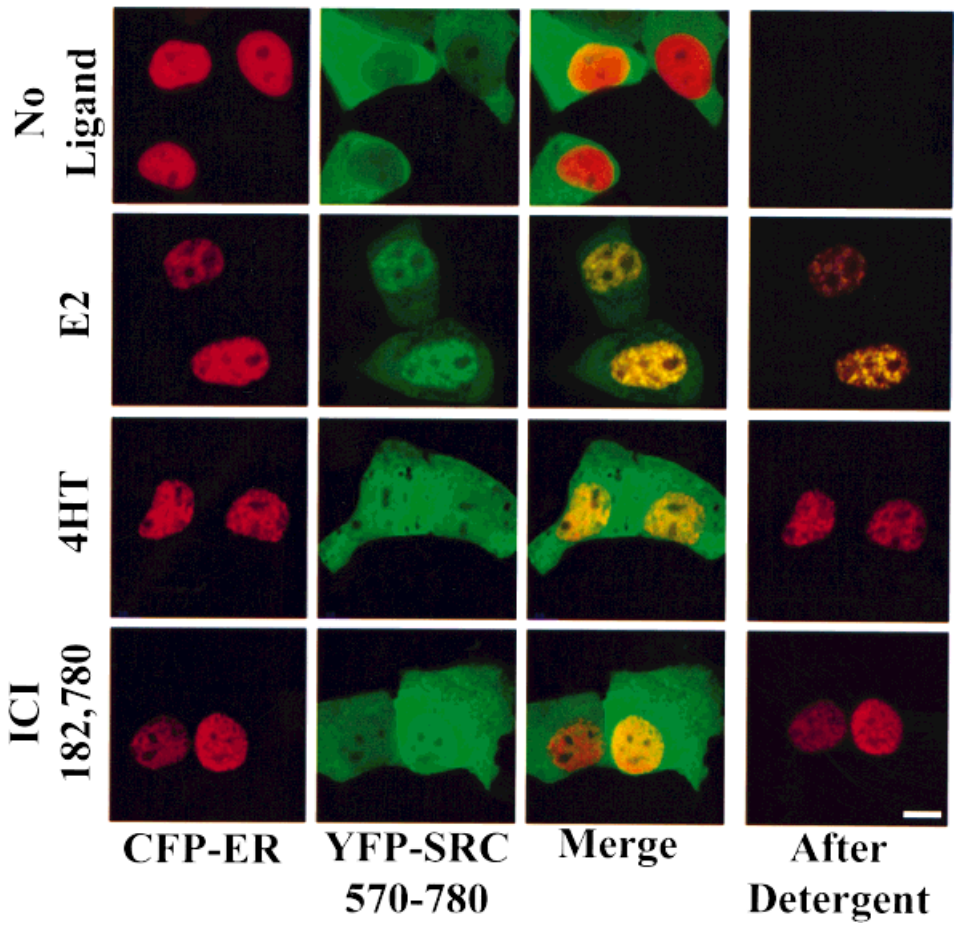

Fig. 1. CFP-ER is insoluble and recruits coactivators following agonist addition. HeLa cells were co-transfected with CFP-ER (red) and a YFP-SRC1 fragment (YFP-SRC570-780; green) containing the LXXLL motifs required for ER interactions. Cells were imaged following treatment for 2 h with vehicle (top row), 10 nM E2 (second row), 10 nM 4HT (third row) or 10 nM ICI 182,780 (bottom row), CFP-ER is shown in pseudo-color red and YFP-SRC570-780 is shown in green. The YFP-SRC570-780 is predominantly cytoplasmic in the absence of hormone but accumulates in the nucleus and co-localizes with CFP-ER only in the presence of E2. In the absence of added ligand, most of the CFP-ER and YFP-SRC570-780 fluorescence was removed following treatment with detergent. Although both agonists and antagonists cause CFP-ER to target the NM, retention of YFP-SRC570–780 occurs only in the presence of both CFP-ER and E2.

the amino terminus of SRC-1. Presumably this smaller fragment of SRC-1 is able to pass through the nuclear pore complex, perhaps through association with other factors, leading to a striking intranuclear accumulation and colocalization with, specifically, agonist-bound CFP-ER.

Although there is a correlation between transcription factor reorganization and transcriptional competence, the functional significance of transcription factor foci remains unclear. Part of this uncertainty is due to the finding that very few of the foci containing ER and other transcription factors colocalize with the active sites of transcription mentioned above [Grande et al., 1997; Stenoien et al., 2000]. A possible explanation for this is that interactions between transcription factors and transcription sites are highly dynamic. The 'snapshots' of transcriptional activity by in situ run ons or direct fixation/immunolabeling approaches only offer a single timepoint view. It is unclear how long the transcription foci are actually active, more importantly, the vast non-localizing transcription factor foci may or may not develop into active transcription sites or, conversely, may have just terminated this activity. Indeed, some of these foci may represent sites of degradation.

A cell line containing multiple copies of the MMTV promoter was recently used to study interactions between fluorescent glucocorticoid

receptors (GFP-GR) and DNA binding sites in vivo [McNally et al., 2000]. Fluorescence recovery after photobleaching (FRAP) revealed that GFP-GR undergoes rapid exchange with DNA on this transcriptionally active locus. This suggests a model in which steroid receptors interact transiently (for seconds) with transcription sites and this is sufficient to initiate transcription. This model may help to explain why only a small fraction of the transcription factors are found at transcription sites when a static image of fixed cells is analyzed by immunofluorescence.

FRAP data analyzing bulk, nuclear CFP-ER, in the presence and absence of different types of ligand, demonstrates that ER can exhibit different types of intranuclear mobility dependent upon its ligand-bound state [Stenoien et al., 2001]. In the absence of any ligand, CFP-ER is highly mobile (recovery half-life of ~0.8 sec) indicative of a molecular complex that makes few if any specific interactions with relatively immobile nuclear components. Following treatment with an agonist, estradiol, or the partial antagonist, 4-hydroxy tamoxifen, CFP-ER mobility is slowed (recovery half-life of ~5–6 sec). As ligands surely cause steroid receptors to shed some proteins and recruit others, the change in mobility may be an extension of the receptor's affinity to insoluble components directly or indirectly. However, since CFP-ER recovers within seconds, this indicates that interactions with the NM are highly dynamic. Other nuclear factors (i.e., splicing factors) that have been shown by biochemical methods to partition with the insoluble nuclear matrix also exhibit this dynamic behavior with relatively fast photobleach recovery rates of seconds [Kruhlak et al., 2000; Phair and Mistelli, 2000].

The rapid exchange rates observed with these nuclear proteins has led to conflicting interpretations on the involvement of the NM in nuclear organization. Two recent commentaries have implied that the seemingly rapid mobility rates demonstrate movement within the nucleus is mostly diffusional with little influence exerted by insoluble nuclear structures [Lewis and Tollerwey, 2000; Pederson, 2000]. An alternative interpretation of these data is that the reduced mobility of nuclear proteins is due to interactions with a nucleoskeleton [Kruhlak et al., 2000; Shopland and Lawrence, 2000; Stenoien et al., 2001]. Supporting this second interpretation is the observation that mobility is slower than expected for GFP and even large macromolecules (up to 580 kD) that are relatively free to diffuse within the nucleus [Seksek et al., 1997]. Since the size of the steroid receptor complexes do not drastically change (~500 kD before and after hormone treatment) [McKenna et al., 1998], this argues that the ligand-specific reduction of mobility is due to interactions with components of the NM. In contrast to ruling out the existence of interactions of transcription factors with the NM, the above studies point out the importance of dynamic vs. static interactions. Recent FRAP studies on agonist-bound ER demonstrate that rapid exchange occurs with target sites in chromatin and the NM. In the presence of the pure antagonist, ICI 182,780 however, CFP-ER is immobilized with little fluorescent recovery over several minutes following the bleach (Fig. 2). The ICI 182,780-bound receptor is detergent insoluble and biochemically partitions completely with the NM fraction [Stenoien et al., 2000] following chromatin removal suggesting that it is bound to some structure other than chromatin. Dual FRAP studies with CFP-ER and YFP-SRC-1 indicate that even individual components of transcription complexes undergo rapid exchange in the presence of agonist [Stenoien et al., 2001].

Our lab has utilized distinct but complementary approaches to study the interactions of steroid receptors with the NM. First, careful biochemical analysis of endogenous ER partitioning in MCF-7 cells demonstrates a dramatic shift from the detergent soluble to the NM-bound fraction within minutes of adding ligand. This solubility shift occurs on the same time scale (min) as foci formation occurs indicating the two events may be related [Stenoien et al., 2000]. To confirm this, we have developed a method to analyze the solubility of fluorescently-tagged steroid receptors and coregulators in real time. This method involves analyzing individual cells before, during, and after the extraction protocol to obtain an unambiguous assessment of the extent of NM association. An important finding from these real-time analyses is that protein expression levels can affect the extent of NM association. In cells where ER is grossly overexpressed, the receptor is largely insoluble regardless of its ligand bound state, whereas in cells that express lower levels of ER, the receptor solubility

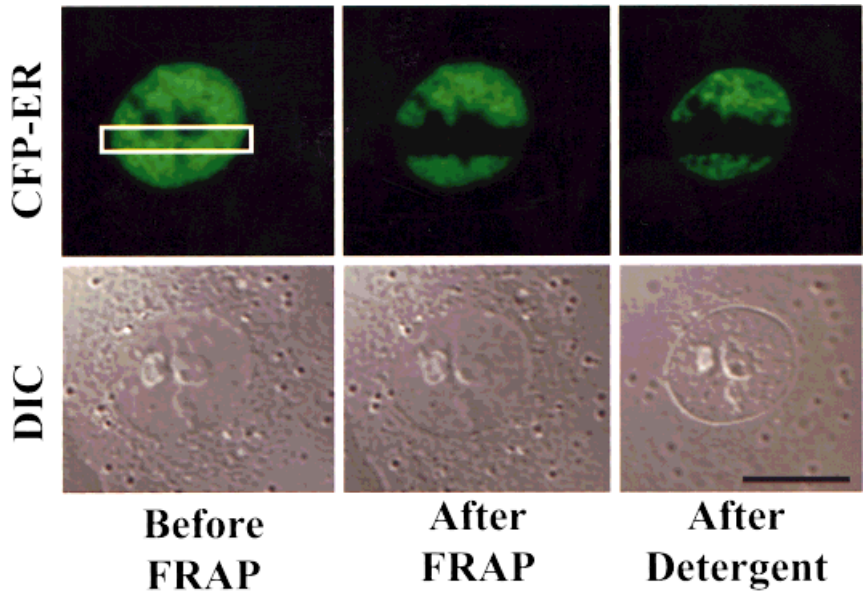

Fig. 2. CFP-ER is immobile and insoluble in the presence of ICI 182,780. HeLa cells transfected with CFP-ER were treated with the ER antagonist ICI 182,780 (10 nM, 20 min, **left panels**). The region denoted by the box was bleached by repeated laser scanning for 2 sec. No fluorescence recovery after photobleaching (FRAP) is observed after 1 min (**middle panels**). A real-time detergent extraction using buffer with 0.5% Triton X-100 was performed to demonstrate the immobile receptor is also insoluble (**right panels**). Bar = 10 microns.

reflects the same ligand dependence as endogenous ER. When the solubility of the ER is analyzed by western blotting techniques, the results can be skewed by the presence of the small number of cells that overexpress large amounts of protein.

These findings point to two important issues relating to NM partitioning studies in general. First, great care must be taken when assessing the NM association of transiently transfected proteins due to potential artifacts caused by overexpression. This reality of transient transfections suggests that re-evaluation of some NM targeting studies may be required. Second, the NM may have a physiological role in the removal of excess and/or misfolded proteins. Several studies have suggested pathways involving ubiquitin and proteasomes that play an important role in steroid receptor function [Alarid et al., 1999; El Khissiin et al., 1999; Nawaz et al., 1999a]. In one report, the E6-AP protein, a ubiquitin-protein ligase, was shown to function as a ligand-specific steroid receptor coactivator [Nawaz et al., 1999b]. As ubiquitin conjugated proteins are processed by the 26S proteasome complex [DeMartino and Slaughter, 1999], this suggests that ubiquitin modified steroid receptors are processed by proteasomes. Inhibition of proteasome function prevents receptor downregulation providing direct evidence of proteasome involvement in steroid receptor turnover. Ligand dependent turnover may be important for transcriptional control by down regulating transcription complexes. Moreover, proteasomes may regulate the dynamic interactions required for transcription as treatment with proteasome inhibitors prevents ER-based transcription even though ER protein levels increase [Lonard et al., 2000]. FRAP analysis also shows that even brief inhibition of proteasome function results in an immobile, NM-bound fraction [Stenoien et al., 2001].

The FRAP studies demonstrate that transcription factors can have different types of interactions with the NM. In the case of estrogen-bound ER, these interactions are much more transient and dynamic than observed with the statically bound ER following ICI 182,780 addition, proteasome inhibition, or ATP depletion [Stenoien et al., 2001]. In each case however, traditional biochemical partitioning assays show both the dynamic and static receptors partition with the NM fraction implying that even transient interactions with the NM are sufficient to retain ER within the nucleus following detergent treatment. The finding that

ligand-bound ER does not diffuse out of the nucleus and remains insoluble even following chromatin removal [Stenoien et al., 2000] suggests that ER interacts with some structure other than chromatin. Also, NM-bound GFP-ER foci undergo little change in their size and distribution during real-time monitoring of the extraction procedure thus indicating NM association is not due to non-specific aggregation of certain proteins [Stenoien et al., 2000].

Targeting of overexpressed proteins to the NM may reflect a physiological role for the ligand-independent partitioning. Immunolabeling studies suggest there is an upregulation of heat shock proteins within the nuclei of some cells overexpressing nuclear proteins, perhaps indicative of an attempt to deal with an increase in misfolded proteins. This upregulation of heat shock proteins is observed with aggregate-forming AR mutations found in a cancer patient [Nazareth et al., 1999], (Stenoien DL, Mancini MG, Patel K, Mancini MG, Weigel NL, Mancini MA, manuscript in preparation) and due to polyglutamine expansion [Stenoien et al., 1999]. In the latter case, co-expression of chaperones inhibit aggregate formation. In addition to upregulation of endogenous heat shock proteins, protein aggregates also sequester proteasome components. A second example of the physiological relevance of this process is found with the Pit-1 mutants mentioned above that can aberrantly associate with the NM. Chaperone and proteasome labeling also suggests a cell stress response (Sharp ZD, Stenoien DL, Mancini MG, Mancini MA, manuscript in preparation). These findings are reminiscent of the cell stress and proteasome response caused by polyglutamine expanded proteins associated with neurodegenerative disorders. Recent photobleaching studies of the Pit-1 mutants indicate that at low protein levels they exhibit similar dynamic behavior as wild-type Pit-1. However, once a threshold level of expression is obtained, the Pit-1 mutants can become immobilized and rapidly accumulate on the NM. Interestingly, unlike several inactivating Pit-1 point mutants, even gross overexpression of wild-type Pit-1 does not lead to increased NM association [Mancini et al., 1999]. These results suggest that the NM may serve as a depository and disposal site for, in particular, misfolded proteins, and this site can be swamped due to overexpression or accumulation of mutant proteins over time.

CONCLUSIONS

Recent data continue to shed light on the interrelationships between nuclear structure and function. Increasingly, new studies support a role of the NM in the organization of the metabolic machineries within the nucleus. This organization may help to ensure that processes such as gene transcription are carried out in an efficient manner. In addition, the NM may serve as a site involved in the disposal of misfolded proteins and clearance of transcription factors following transcription. Also, in contrast to the static images obtained using fixed microscopic specimens and biochemical portioning assays, live microscopy and FRAP demonstrate that many nuclear factors make dynamic interactions with the nuclear substructure. These latest results suggest a model in which the NM regulates both the spatial and temporal distribution of nuclear proteins to ensure that these proteins function efficiently. Furthermore, mutations that disrupt the localization and/or dynamics of NM-associated proteins may have consequences related to human diseases.

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