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Compartmentalization of regulatory proteins in the cell nucleus☆

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Abstract

The cell nucleus is increasingly recognized as a spatially organized structure. In this review, the nature and controversies associated with nuclear compartmentalization are discussed. The relationship between nuclear structure and organization of proteins involved in the regulation of RNA polymerase II-transcribed genes is then discussed. Finally, very recent data on the mobility of these proteins within the cell nucleus is considered and their implications for regulation through compartmentalization of proteins and genomic DNA are discussed.

Keywords: Cell nucleus; Regulatory proteins; Genomic DNA

1. Introduction

Studies that incorporate subnuclear localization of catalytic and regulatory proteins within the cell nucleus are rapidly increasing in number. This is due to both an increase in the availability of antibody, and more recently, fluorescent protein reagents, and an increase in the use of confocal microscopy. The virtually universal finding is that proteins are not homogeneously distributed within the nuclear volume, but rather, are enriched at steady-state levels in discrete nuclear structures. The hypothesis that there is a coordinated organization and spatio-temporal regulation of nuclear processes is now being explored.

A structural basis for compartmentalization of function within the cell nucleus originated with biochemical and ultrastructural studies of Berezney and Coffey [1–4]. These authors developed a protocol for isolating a chromatin-depleted residual nuclear structure termed the 'nuclear matrix', and were the first to propose that this structure represented a framework for the organization of nuclear function [5,6]. In a pioneering study, they demonstrated that newly synthesized DNA was highly enriched in this chromatin-depleted nuclear fraction [1]. Ultrastructural parallels with the previously defined RNP-reticulum component of the interchromatin space [7,8] implied a physiological relevance to this biochemically isolated nuclear fraction [4].

Compartmentalization is a process that is well-established within the cytoplasm. Generally, however, it has been associated with membrane-bounded cytoplasmic organelles. The absence of intranuclear membranes has contributed to the skepticism towards compartmentalization having a role in nuclear regulation. Recent studies of the role of the cytoskeleton in signal transduction mechanisms, however, clearly indicate that a membrane boundary is not the only mechanism of sequestering catalytic molecules within a defined space. An excellent example is the function of SMAC complexes, which are localized cytoskeletal assemblies that concentrate signal transduction molecules during lymphocyte activation. The assembly of SMAC complexes is essential for a complete activation response and is believed to play a quantitative rather than qualitative role in signal transduction [9].

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With the advent of GFP technology and microscopy techniques to image protein and structural dynamics in living cells, one is beginning to appreciate the dynamic nature of compartments in the cell nucleus [10–12]. All structures so far examined have shown some evidence of positional dynamics [11,13–21]. However, the potential mobility of many structures, defined by their size and the 'porosity' of the nucleoplasm, is far greater than their actualized mobility [21]. This implies underlying organization in the generation of compartments. It has also become clear that the constituents of compartments, themselves, are dynamic. Thus, these compartments are dynamic but show steady-state accumulations of specific proteins whose composition may be altered depending on the metabolic state of the cell [10,21].

In this review, one will consider the evidence for a regulatory influence of compartmentalization on RNA polymerase II-mediated transcription. Focus will be on the organization of chromatin, its relationship to the spatial organization of transcription factors, and how the process of reversible histone acetylation may be a useful model for understanding the relationship between organization and function.

2. Higher-order chromatin structure during interphase

Biochemical studies have demonstrated differences in the structure of active and competent chromatin from repressed and heterochromatin. This is predominantly based on sensitivity to exogenous probes such as nucleases. The underlying basis of this difference, however, is far from clear. The conventional interpretation is that nuclease-sensitive chromatin reflects unfolding of the 30 nm fiber; nuclease-insensitive chromatin is folded to 30 nm fibers and higher levels of packing. Cytological studies do not support the existence of significant amounts of chromatin unfolded below 30 nm fibers. Rather, they are more consistent with the organization of active chromatin into fibers greater than 30 nm but still less compact than heterochromatin which maintains a state close to its metaphase organization [22].

Estimation of DNA packing ratios in transcriptionally active genes of human cells indicates that even active genes are quite compact [22,23]. The nature of this compaction was revealed in recent high resolution fluorescence and electron microscopy studies, where interphase chromosomes are dominated by substructure consisting of 100–200 nm fibers [20,24–27] (Fig. 1). The high packing density of genes within nuclear space produced by both chromatin folding could provide a basis for spatially organizing the genome. Consistent with this hypothesis, the labelling of sites of transcription and their localization by electron microscopy has demonstrated that transcription occurs at the surface of condensed higher order (>30 nm) chromatin fibers [28–31].

In recent years, understanding of the higher-order organization of the genome during interphase has significantly advanced. Structural studies of interphase chromosomes have revealed that they occupy discrete territories [20,32–34] and, in some tissues at least, preferred nuclear positions [35,36]. The differentiation of chromosomes into gene-rich R-bands and gene-deficient G-bands, maintained in interphase chromosomal territories, provides a basis for compartmentalizing these two classes of DNA sequences within interphase nuclei [37]. Interestingly, R-bands and G-bands of individual chromosomes distribute differently within nuclear space, with the gene-rich R-bands appearing as foci that are predominantly found near the interior of the cell nucleus and the G-bands located at the periphery [38]. The organization of transcriptionally active and competent chromatin detected using antibodies to highly acetylated histone species (Fig. 2) also shows this subnuclear organization. Highly acetylated chromatin is depleted on the periphery of fibroblast nuclei. Instead, highly acetylated chromatin is found in foci and small fibers located in particular abundance on the periphery, but do not encroach upon the interior, of nuclear speckles [39]. Electron microscopy of nuclear speckles and their surrounding nucleoplasm shows that the chromatin in the vicinity of speckles is organized into higher-order fibers that are physically separated from the speckle domain by small regions of intervening nucleoplasm [39,40]. It is essential to define the nature of the intervening space between chromatin and nonchromatin elements of the cell nucleus and deter-

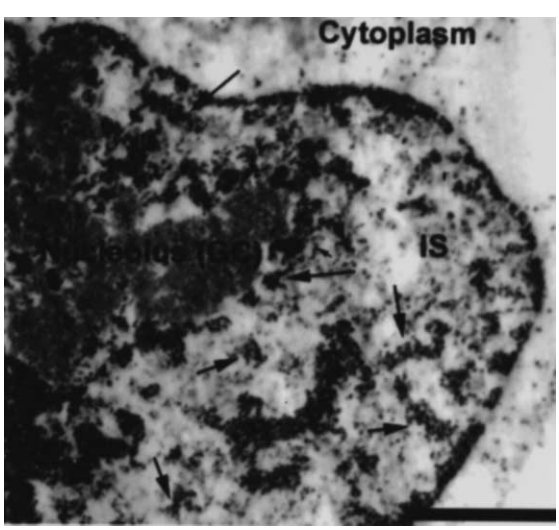

Fig. 1. Chromatin organization in interphase nuclei. A TEM image of an Indian muntjac fibroblast cell nucleus is shown. The arrows indicate the positions of chromatin fibers of 100–200 nm diameter. IS indicates interchromatin space. GC indicates granular component. The scale bar represents 1 μm.

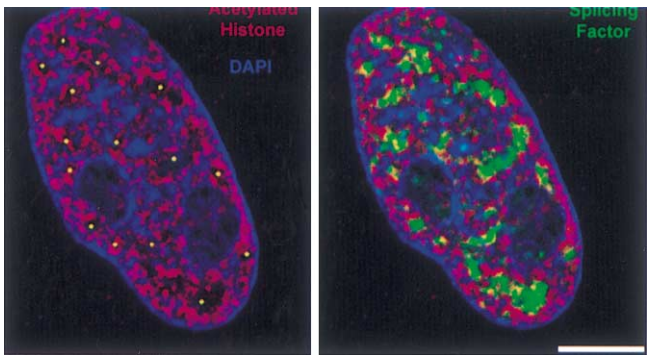

Fig. 2. Subnuclear distribution of chromatin undergoing histone acetylation. An Indian muntjac fibroblast cell was treated with a histone deacetylase inhibitor for 60 min. Following treatment, the cell was fixed and immunofluorescently stained with antibodies to highly acetylated histone H3 (acetylated histone), SC-35 (splicing factor), and DAPI, which detects total DNA. The scale bar represents 5 μm.

mine whether or not there are structural components that integrate the nucleoplasm to maintain this organization.

2.1. Electron microscopy of the interchromatin space

Understanding of the ultrastructure of the cell nucleus has been strongly influenced by the development of the EDTA regressive staining technique by Bernhard [7,8]. This technique stains RNA with uranyl acetate preferentially to chromatin. This led to the identification of 'perichromatin fibrils', structures that are believed to represent nascent RNAs. These are a highly heterogeneous class of structures that range in diameter from 3 to 20 nm [8]. In the original application of this technique, rigorous controls were done that included digestion with nucleases and proteases as well as chemical modification. The presence of nascent RNA in perichromatin fibrils is supported by numerous studies that demonstrate labelling of perichromatin fibrils with metabolic probes for rapidly synthesized RNA and with antibodies recognizing hnRNP proteins [31,41,42].

In more recent applications of the EDTA-regressive staining procedure, it is common to identify any fibrillar component of the nucleoplasm that is well contrasted as a perichromatin fibril. This gives the impression of an interchromatin space dominated by perichromatin fibrils. An approach that is both quantitative and capable of compositional analysis but does not rely upon the ability of uranyl acetate stain to deposit on structures is electron spectroscopic imaging (ESI) [40,43–45]. ESI allows quantitative assessment phosphorus and nitrogen distributions. Because both RNA and DNA are rich in phosphorus, nucleoprotein structures of the nucleoplasm give both strong phosphorus and nitrogen signals. Early applications of this technology demonstrate that the EDTA-regressive staining method does not distinguish between protein and RNA within the nucleoplasm [46].

ESI has also been applied to determining the composition of the interchromatin space [39,40]. Using this approach it was demonstrated that most of the RNA is found as particles associated with a protein architecture and that RNA is a minor component, by mass, of the interchromatin space (Fig. 3). It is interesting to note that one of the original Berezney and Coffey papers describing the 'nuclear matrix' noted the excellent morphological similarities between the ribonucleoprotein reticulum identified by EDTA-regressive staining and the isolated nuclear matrix [4]. The ultrastructural studies using ESI indicate that this ribonucleoprotein reticulum is actually dominated by a protein karyoskeleton that has the potential to function in the organization of both chromatin and nonchromatin structures in the coordination and compartmentalization of RNA polymerase II transcription.

3. Chromatin dynamics in living cells

Chromatin in fixed cells appears to be organized into higher order fibers that are typically between 100 nm and 200 nm in diameter. Correlative electron and fluorescence microscopy has demonstrated that highly acetylated chromatin is found within these higher-order fibers [47] indicating that these regions are in a transcriptionally competent conformation. Recently, experimental support for higher-order organization of transcribing chromatin in living cells has been obtained [22,48].

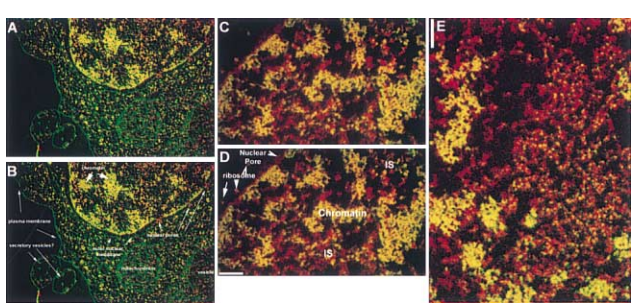

Fig. 3. Electron spectroscopic imaging (ESI) imaging of the interchromatin space. Panels A and B demonstrate the use of ESI for analyzing the composition of different biological structures. Net phosphorus (green) and net nitrogen (red) quantitative spatial maps were obtained for a lymphocyte cell postfixed with osmium tetroxide to preserve biological membranes. In Panel B, individual structures are labeled. Membranes, comprised of phospholipids that are rich in phosphorus but depleted in nitrogen, appear green, nuclear pores, are depleted in phosphorus due to an absence of membrane or nucleic acids, but rich in nitrogen due to the presence of protein, appear red. Structures that are comprised of both phosphorus-rich molecules and nitrogen-rich molecules (e.g. DNA and RNA) appear yellow. Panels C and D show a high magnification view of the interchromatin space and Panel E shows a high magnification view of an interchromatin granule cluster. The interchromatin space and interchromatin granule cluster are predominated by a protein signal (red) within which nucleoproteins (yellow) are embedded. The scale bar represents 250 nm in Panel D and 100 nm in Panel E.

Studies on the positional dynamics of chromatin indicate that it is considerably immobile over short periods of time (s) but does show positional dynamics over longer times [13,15,16,19,20,25,26]. Moreover, direct evidence has been obtained for transcriptional activity in higher-order fibers [22]. In cell lines stably transformed with histone-fluorescent protein constructs, heterochromatin exhibits high positional and conformational stability whereas more dynamic behavior, particularly conformational rather than positional dynamics, is seen for extended higher-order fibers (Lever et al., unpublished observations). Nonetheless, the chromatin density throughout the nucleus is not high enough to generate steric limitations to chromatin movement and yet the restriction of chromatin movement has been demonstrated in both dynamics studies and fluorescence recovery after photobleaching experiments.

The significance of the observations of chromatin folding and chromatin mobility in interphase nuclei is that they can be directly related to the potential benefits of spatio-temporal organization of regulatory factors. The organization of chromatin into higher-order fibers, the organization of these fibers into discrete chromosomal territories, and the restricted mobility of the chromatin within the cell nucleus provides an appropriate substrate for spatio-temporal organization to have regulatory benefits.

4. Subnuclear organization of transcription factors

If spatio-temporal regulation occurs, it would require the subnuclear organization of transcription factors. The characteristic distribution of a transcriptional regulatory protein is localization to several hundred discrete foci located throughout the nucleoplasm [49] (Fig. 4). This organization has recently been documented in living cells as well [50–53]. A simple explanation for the enrichment of transcription factors into foci is that this reflects a clustering of binding sites within chromosomes and the folding of chromatin into 100–200 nm fibers. Although, in the case of steroid hormone receptors, their appearance correlates with transcriptional activation [50,51], studies that have examined codistribution of transcription factor foci with sites of transcription or RNA polymerase II show only minor overlap [49,54,55]. Additionally, evidence has been provided that these domains can occur in regions devoid of chromatin [39]. Thus, most of these foci probably do not correspond to chromatin-associated transcription factors. An alternative explanation, favored by the existing data, is that foci represent nonchromatin structures that function in the regulation of nucleoplasmic concentrations of factors and/or the assembly of multimolecular transcription factor complexes.

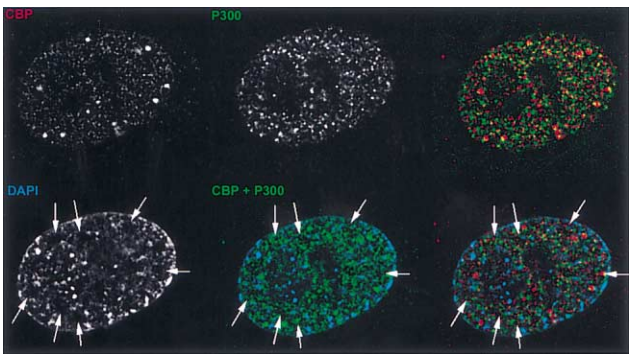

Fig. 4. Subnuclear organization of the CBP and P300 histone acetyltransferases. An MRC-5 human lung fibroblast cell was stained with antibodies recognizing the N-terminus of CBP, P300, and DAPI. The image shows a projection of digitally deconvolved serial sections spanning the depth of the nucleus. Most of the CBP and P300 are localized to small foci. CBP also shows a smaller number of very bright foci that correspond to PML bodies. The colors of the text represent the colors of the individual proteins and DAPI in the merge figures. The arrows indicate the positions of chromatin-depleted regions likely to contain nuclear speckles.

We suggest that transcription factor-enriched foci are specialized nonchromatin sites that may function in two ways. First, we propose that they function to concentrate specific nuclear proteins to facilitate the assembly of multimolecular regulatory complexes. Nuclear speckles, enriched in splicing factors, have been proposed to function in this manner [10]. It is quite controversial whether or not pre-mRNAs enter nuclear speckles [39,41,56–63] but it is very clear that most or all splicing factors enter these domains. In the case of transcription factor foci, two studies indicate that they may function similarly. Transcriptional coactivators are recruited to ER-containing foci upon induction with estrogen [53]. Also, it has been found that HDAC3, which is found in a complex with HDAC4, is recruited to a subset of HDAC4 containing foci (Fischle et al., unpublished observations) (Fig. 5). Second, we propose that these foci function to control both global and local nucleoplasmic concentrations of assembled complexes.

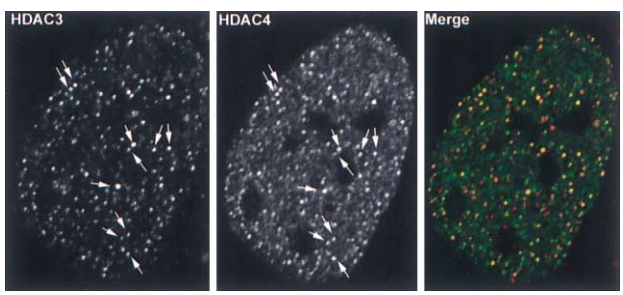

Fig. 5. Colocalization of HDACs 3 and 4 in discrete nuclear foci. A HeLa cell was transfected with a FLAG epitope-tagged HDAC4 expression vector and costained for endogenous HDAC3. The arrows indicate the positions of discrete foci that contain both HDAC3 (red in merge image) and HDAC4 (green in merge image).

Evidence to support this second function is presented in several of the following sections.

5. The histone acetylation process as a model to examine spatial regulation of activity

Histone acetylation, an essential component of gene regulation, is a reversible reaction that is catalyzed by two opposing activities, histone acetyltransferases (HATs) and histone deacetylases (HDACs). Elevated levels of histone acetylation have long been correlated with transcriptional activity, but the identification of HATs and HDACs as previously known transcriptional regulators consolidated this correlation [64–72].

There has been a lot of attention paid recently to the targeting of specific promoters and enhancers. However, earlier studies demonstrated that histone acetylation correlates with DNase I sensitivity [73–76] and that this sensitivity extends well beyond promoters and enhancers through a large length of DNA that typically include several kbps of flanking sequences as well as coding sequences [77–79]. Thus, although multiply acetylated histones are confined to only a small portion of the genome, they are more pervasive than can be accounted for by specific targeting to promoter and enhancer regions. Interestingly, these multiply acetylated species have very short half-lives (several minutes) compared to the lower acetylated species (several hours) and thus, both HATs and HDACs must be targeted to these regions of the genome [80]. The availability of antibody reagents to examine the subcellular distributions of both the enzymes responsible for acetylation and deacetylation, antibody reagents to both substrates and products of each reaction, and specific inhibitors of histone deacetylases make this process an ideal model system for testing the possibility that the targeting process is influenced by spatially organizing both enzymes and chromatin.

There are many potential mechanisms for orchestrating differential acetylation on this level. One mechanism, proposed several years ago by Waterborg and Matthews [81] and later by Davie and Hendzel [80,82], involves the immobilization of the enzymatic machinery on the nuclear matrix. The experimental demonstration that HATs and HDACs were predominantly found in nuclear matrix fractions is consistent with a compartmentalization of the histone acetylation process [82–85]. Direct evidence for spatial organization of highly acetylated chromatin, HATs, and HDACs can be demonstrated by indirect immunofluorescence microscopy. Dynamic histone aceetylation, the type associated with transcriptionally active chromatin, does not occur on the periphery of fibroblast nuclei but rather is enriched on the periphery of splicing factor-enriched domains often termed nuclear speckles [39]. Additionally, the enzymes responsible for histone acetylation are enriched on the exterior portion of nuclear speckles, partially overlapping adjacent dynamically acetylated chromatin. Evidence for localization of regulatory factors in a non-chromatin domain indicates that the subnuclear targeting of such factors is independent of chromatin to some degree.

If foci exist independently of chromatin, are they functionally required for histone acetyltransferase and deacetylase activity? This was recently addressed by examining the activity of HATs and HDACs through mitosis [86]. As cells progress through prophase, foci are dispersed and HATs and HDACs collect in the interchromosomal spaces. The enzymes remain dispersed throughout mitosis until early G1, where foci reassemble. The dispersion with a complete inactivation of both HAT and HDAC activity in situ and the reassociation of these enzymes with foci correlates with their reactivation. Importantly, extracts prepared from mitotic cells indicate that both in vitro HAT and in vitro HDAC activity is maintained at interphase levels throughout mitosis. Thus, there is a correlation between the assembly of these factors into foci and their ability to acetylate and deacetylate chromatin in cells but not their catalytic activity in vitro.

6. Positional mobility of foci enriched in transcription factors

If foci enriched in transcription factors are important in the spatial control of factor availability, they must be positionally stable over relatively short periods of time (min) but capable of either movement or dissolution and formation in new positions over longer periods of time. Alternatively, it has been suggested that nuclear domains may be able to diffuse through the nucleoplasm [12]. It has been demonstrated that structures approximately twice the size of transcription factor foci can be highly mobile within the cell nucleus [21]. Thus, it would be anticipated that if these structures are not tethered, they would be continuously and rapidly moving. In order to address this question, 2-D timelapse microscopy experiments were performed on cells stably transfected with ER-GFP and 3-D timelapse imaging of cells stably transfected with CBP-GFP and AP-2-GFP. Over short time intervals (s), foci are positionally stable, not freely mobile in the cell nucleus [87]. Over longer time periods, these foci show positional dynamics and are capable of fusing with nearby foci. No evidence of the formation of new sites or the dissolution of old ones has been obtained. Instead, remodelling appears to occur primarily through translocation of existing foci. Importantly, foci show more positional mobility than chromatin supporting conclusions based on colocalization data that foci are not sites of tran-

scription factor binding to chromatin. The positional mobility of foci is consistent with the proposal that these structures function to assemble multiprotein complexes and/or regulate the concentrations of factors locally.

7. Mobility of transcriptional regulators within the cell nucleus

If these foci function in the manner that we propose, then they must represent steady-state compartments that are constantly exchanging factors with the surrounding nucleoplasm. This can be directly determined using fluorescence recovery after photobleaching (FRAP). In FRAP experiments, a region expressing a fluorescently-tagged molecule is exposed to sufficiently high energy laser light to irreversibly eliminate fluorescence from the molecules in the exposed region. The exchange of molecules into the photobleached region is then monitored by timelapse microscopy. Splicing factors move into and out of the steady-state speckle compartments very rapidly [21,88] with an estimated residency time of less than 50 s [88]. Thus, nuclear speckles are constantly exchanging at least some of their components with the surrounding nucleoplasm.

Following this precedent, the mobility of several transcriptional regulators have been examined [87]. The details of the inducible regulator, the estrogen receptor, will be discussed separately in sections that follow. Constitutively active regulators that were examined included the AP-2 DNA binding transcriptional activator, the PCAF and CBP coactivators/HATs, and the HDAC-1 and HDAC-4 corepressors/histone deacetylases. Both stable and transient transfectants were examined for AP-2 and CBP; PCAF and HDAC-1 were examined as transient transfectants. AP-2, when expressed as a GFP chimera, consistently assembled into foci and moved at rates at least one hundred-fold slower than free diffusion. Nonetheless, the factor clearly moved into and out of individual foci more rapidly than the foci themselves move throughout nuclear space. The CBP protein produced the most interesting results. The stable transfectants were exclusively found to express the chimeric protein in a homogenous distribution outside of nucleoli, where the factor was excluded. In FRAP experiments, this population of CBP-expressing cells showed mobilities very similar to that of the GFP alone and were therefore consistent with free diffusion. The mean diffusion coefficient was 5.5 μ^2/s, approximately 10-fold slower than GFP alone. In transient transfectants, two populations of cells were observed. Some cells expressed CBP-GFP with enrichment in foci and PML bodies while others expressed it diffusely. FRAP experiments demonstrated that these two populations of cells behaved quite differently. The diffusely expressing cells behaved identical to the stable transfectants whereas the cells that showed evidence of foci also showed considerably reduced mobility of the CBP protein and had diffusion coefficients between 0.4 and 2.0 μ^2/s. Since all of the factors examined distributed in foci when the endogenous protein was detected by indirect immunofluorescence of nontransfected cells, it was believed that the physiological behavior of the native protein is reflected by the subset of cells expressing CBP-GFP in foci.

The absence of this population in the stably tranfected cells suggests that the association of CBP with foci can be regulated and that the cells can adapt to overexpression by deregulating localization. This is consistent with an important role of foci in the regulation of protein function and, interestingly, serves as a precedent for a transient transfection more accurately reflecting the physiological behavior of a protein than a stable transfection (since the distribution of the native protein is focal in all cell types examined). PCAF, HDAC-1, and HDAC-4 were predominantly expressed as freely diffusible proteins and therefore did not properly target. Interestingly, FLAG-tagged HDAC-4 [89], but not GFP-tagged HDAC-4, localizes to foci. This indicates that in some instances the GFP tag can interfere with the proper subnuclear targeting of a factor.

A final and important result observed in the study of the mobility of the HDAC-4 protein is that enrichment in steady-state compartments does not necessarily mean that the mobility of a protein into and out of these domains is significantly retarded. Overexpression of HDAC4 results in the generation of relatively large intranuclear domains [21,90,91]. These domains are not observed in nontransfected cells and, unlike all physiological compartments examined so far, are freely mobile within the nucleoplasm. Interestingly, when these domains are photobleached, they recover at rates the same as or very close to the recovery of the nucleoplasm, where the protein is freely diffusible (our unpublished observations).

8. There are immobile proteins within the cell nucleus

We have discussed how many transcriptional regulators move into and out of structures quite rapidly. It has been suggested that structures that are visible by fluorescence microscopy represent dynamic assemblies of molecules based on weak interactions. Where the requirement for activity is high, these domains accumulate in large enough quantities that they form visible domains [92]. Although there is likely some element of truth to this, it is difficult to explain, for example, the translocation of splicing-factor enriched structures from IGCs to nearby sites of transcription [17,93] based solely on dynamic associations. For example, at least

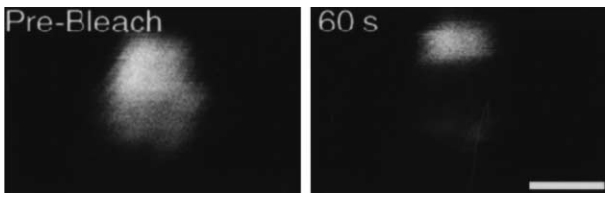

Fig. 6. Photobleaching of Sp100 in a PML body. A cell expressing an Sp100-GFP chimeric protein localizing to PML bodies was selected and a single PML body was photobleached through the center of the structure. The image shows a pre-bleach image and an image collected 60 s after photobleaching. The scale bar represents 200 nm.

some splicing factors move into and out of IGCs quite readily [21,88]. A dynamic interaction model would predict that molecules are concentrated in IGCs, assemble into functional complexes, dissociate from IGCs and diffuse through the nucleoplasm until they reach substrate. The directional translocation of discrete structures from IGCs to nearby sites of transcription represents a relatively slow mechanism of moving factors around (relative to free diffusion). The fact that translocation of factor-enriched structures is observed and that the appearance and disappearance of discrete domains is not observed over the time-courses studied to date, indicates that such a model is an oversimplification of what actually takes place. Instead, these results predict that there are structural components of domains that have a much higher half-life within a given structure and are responsible for the transient retention of other factors.

We have been examining the structure, composition, and dynamics of PML bodies. PML bodies are structures that are functionally implicated in promyelocytic leukemia. They are also the nuclear sites from which many viral genomes initiate productive viral replication centers [94,95]. In noninfected cells, these domains are positionally stable (unpublished observations) and have been demonstrated to be components of isolated nuclear matrices [96]. These domains have also been demonstrated to accumulate the histone acetyltransferase/transcriptional coactivator CBP [46,97,98]. Despite the accumulation of CBP in PML bodies, these domains do not contain chromatin or RNA in their interior [46]. The dynamics of association of three components of PML bodies using FRAP techniques have been examined [99]. CBP moved into and out of PML bodies with similar kinetics to its movement throughout the remainder of the nucleus. A complete replacement of CBP in a PML body is observed in less than 5 minutes. In contrast, Sp100 and PML have very long half-lives within PML bodies. It was possible to bleach subregions of these 500 nm or less domains and observe the persistence of the bleached region (Fig. 6) for at least 15 min. This demonstrates that Sp100 and PML are essentially positionally fixed within PML bodies and serve as a precedent for proteins with the mobility characteristics of structural components of a nuclear domain. Proteins such as these may comprise the fixed binding sites for the more transient components of steady-state compartments. Consistent with this, overexpression of the PML protein results in increased recruitment of endogenous CBP to PML bodies [99].

9. The estrogen receptor and other inducible regulators

One of the central questions that remains to be answered is whether or not the affinity of association of regulatory factors with steady-state compartments can be regulated. The relocalization from a diffuse distribution to an enrichment in foci upon induction of steroid hormone receptors [50,52] suggests that this might occur. One might expect that the diffuse distribution before induction correlates with a freely diffusible protein and the enrichment in foci following induction correlates with a protein of reduced mobility. Although this is supported by the experiments with the CBP protein, it has been previously demonstrated that the pattern of distribution is not a predictor of mobility. The ASF splicing factor is diffusely distributed outside of speckles and yet there is very little or no difference between the mobility through the nucleoplasm and the mobility into and out of nuclear speckles; both are significantly reduced compared to free diffusion [21].

The subnuclear distribution of the estrogen receptor has been followed in live cells using GFP fusion proteins. The estrogen-receptor, like several other ligand-inducible transcription factors [50,52,54,100] are diffusely distributed or cytoplasmic prior to induction. Upon addition of estrogen or tamoxifen, the ER-alpha redistributes to discrete foci [52,54] commonly observed for consitutively active transcription factors [49]. Although there is a clear correlation between organization and ligand-binding, is this reflected by changes in mobility?

To answer this question, FRAP experiments were once again performed (Fig. 7). Despite the diffuse distribution of the estrogen receptor under nonactivating conditions, the estrogen receptor-GFP chimera had a diffusion coefficient of 3 μ^2/s, slower than predicted for a freely diffusing monomer or dimer. Thus, even in the absence of ligand and obvious subnuclear compartmentalization, the estrogen receptor does not behave as a freely diffusible protein. When cells are treated with estradiol, the estrogen receptor slows substantially more, moving at a rate of only 0.45 μ^2/s, approximately 100-fold slower than free diffusion. Interestingly, tamoxifen also slows the movement of the estrogen receptor, although not as dramatically as estradiol. In cells treated with tamoxifen, the estrogen receptor moves at

0.98 μ^2/s. Interestingly, the pure antagonist, ICI 182870, results in a mobility that is at least 5-fold slower than that observed in the presence of estradiol. In addition, the amount of estrogen receptor immobile during the course of the experiment is significantly increased [87].

The observed mobilities in the presence and absence of ligand correlate well with biochemical studies. In the absence of ligand, most of the estrogen receptor is readily extracted from nuclei [54,101–103]. In the presence of ligand, most of the estrogen receptor is retained in nuclear matrix preparations [54,103–105]. Similarly, tamoxifen also induces the association of the estrogen receptor with nuclear matrix fractions [54]. The results would predict quantitative differences in the amount of extractable estrogen receptor in the presence of tamoxifen compared to estradiol. Although not quantitated, the data presented in Stenoien et al. [54] is consistent with this prediction.

In summary, experiments with hormone-inducible transcriptional regulators indicate that induction correlates with a recruitment of the receptors to focal compartments within the nuclei. In turn, this correlates with a change in the nuclear mobility of the receptors. Thus, the associations of transcriptional regulators with immobile acceptor sites in the nucleus can be regulated in response to phsyiological signals. The critical question that remains is what is the function of this compartmentalization?

10. Determining the function of compartmentalization

Demonstrating a function to subnuclear targeting is not trivial. Although very few subnuclear targeting domains have been identified, the RS-repeat found in a subset of splicing factors represents a well-characterized example. The RS-repeat of the Drosophila suppressor-of-white-apricot and transformer alternative splicing regulators was found to be necessary for targeting of these proteins to nuclear speckles [106,107]. The replacement of this domain with a simple nuclear localization signal had a quantitative, but not qualitative, effect on alternative splice site selection in vivo [106]. However, RS-repeats have been shown to be functionally important in vitro and both RNA binding activity and protein–protein interaction activity have been localized to RS-repeats and shown to be essential for in vitro function. In addition, RS-repeats have been shown to function as nuclear localization sequences. Subsequently, RS-repeats have been shown in mammalian cells to be essential and sufficient for speckle targeting in some SR-proteins but not others [108] and similarly, their importance in vitro is protein-dependent. Thus, given both the functional redundancy in some splicing factors of this well-characterized subnuclear targeting domain and the multiple functions ascribed to this domain, the simple identification of subnuclear targeting sequences will not necessarily directly lead to a determination of the function of subnuclear targeting.

11. Nuclear matrix targeting sequences in transcription factors

Several protein domains that may be responsible for directing subnuclear targeting in transcription factors have also been identified. Consistent with the ambiguities of the RS-repeat, some of these domains have been clearly shown to have other functions. For example, the homeodomain of the LIM-3 protein Lhx3 functions as a nuclear matrix targeting signal [109]. Similarly, nuclear matrix targeting of the glucocorticoid receptor requires both the DNA binding domain and a tau2 transactivation domain [110–112]. Interestingly, the tau2 transactivation domain interacts with a novel protein found in both focal adhesions and nuclear matrix preparations [110]. The protein, Hic-5, positively stimulates glucocorticoid-mediated transcription and is speculated to be a protein capable of transmitting signals from focal adhesion complexes to the cell nucleus [110]. Nonetheless, these investigators previously found that the tau2 domain could be substituted with the

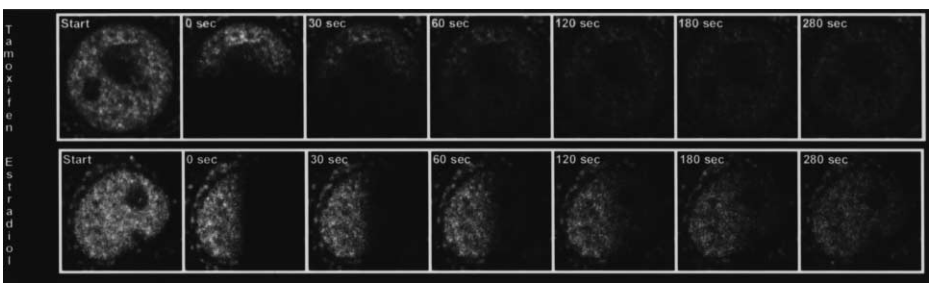

Fig. 7. Photobleaching of the estrogen receptor. An MCF-7 breast cancer cell line was stably transfected with an estrogen receptor alpha-GFP protein. The top panels show a photobleaching experiment performed following treatment with 4-OH tamoxifen and the bottom panels show an experiment performed following treatment with estradiol.

acidic activation domain of VP-16 to mediate nuclear matrix targeting [112]. Finally, the POU-specific domain of the pit-1 transcription factor has been demonstrated to function as a dominant nuclear matrix and subnuclear foci targeting sequence [113]. Interestingly, there are naturally occurring mutations that are implicated in genetic disease and alter the transactivation potential of the POU-specific domain. These mutations, as well as additional mutations generated in vitro, correlate with a greater fraction of the pit-1 protein partitioning with the nuclear matrix [113]. This suggests that dynamic association with the nuclear matrix is essential for pit-1 to function as a transcriptional activator. However, the POU-specific domain has also been reported to be involved in sequence-specific DNA binding [114–116] as well as specific protein-protein interactions believed to occur on chromatin during transcriptional activation [117,118]. The multifunctional properties of the domains identified as nuclear matrix targeting signals in these proteins highlight the potential pitfalls in using deletion analysis to identify domains that are responsible for subnuclear targeting to evaluate the functional role of compartmentalization in vivo.

One nuclear matrix targeting signal has been identified that has not been described previously in the literature as having other functions. This is a 31 amino acid sequence that was first identified in the AML-1B protein [119]. The crystal structure for this domain has recently been solved [120]. The domain is structured as two loops joined by a flexible U-shaped region. Interestingly, this domain shows sequence and function conservation amongst the AML-2 and AML-3 members of this family of transcription factors [120] and is deleted in a naturally occurring translocation found in acute myelogenous leukemia that fuses the ETO protein to AML-1 [121].

In summary, although the identification of protein domains that direct proteins to specific nuclear foci is a useful approach in dissecting the function of subnuclear localization, interpretation of the physiological influence of subnuclear compartmentalization is complicated by the demonstrated or potential multifunctionality of these regions of proteins.

12. HSV-1 infection as a model to manipulate organization and study the effect on function

The potential of multiple functions for individual protein domains involved in subnuclear targeting complicates the direct approach of identifying subnuclear targeting sequences, deleting them, and then assaying the effect on function using biochemical and genetic assays. How then, can one test the function of compartmentalization without deleting protein domains that function to direct a protein to subnuclear compartments? One approach is to generate new compartments in the cell nucleus that will compete with established compartments for targeted proteins.

Fortunately, nature has provided us with an easy way to do this. Many viruses, upon infection, form large nuclear compartments that exclude chromatin. For example, during herpes simplex virus infection, large intranuclear compartments are generated that recruit RNA polymerase II and this recruitment correlates with the repression of RNA polymerase II transcription of host chromatin. Unfortunately, the multifactoral nature of RNA polymerase II transcription initiation in eukaryotic cells complicates a simple interpretation of this data. In other words, transcription could be inactivated because the complexes necessary to potentiate transcription do not form due to different nuclear localizations or it could be inactivated because, although these complexes are present, all or some of them are not available outside of the viral compartments.

As was discussed in detail in a preceding section, histone acetylation is an ideal process to test the functional significance of compartmentalization. Reagents are available to the individual HATs and HDACs as well as to the substrates and products of both reactions. Importantly, HAT activity is present in the individual proteins and, unlike transcription, does not require accessory proteins for activity (although substrate specificity may be modulated by additional proteins). Furthermore, the ability to selectively inhibit the deacetylation reaction provides a tool for measuring both ongoing (rather than steady-state) acetylation and deacetylation. Thus, the tools are available to determine whether or not the histone acetylation process is altered. The key question, then, is whether or not HATs and HDACs are recruited to viral compartments. This is particularly true in light of the fact that HSV-1 DNA is not associated with histones.

In preliminary experiments, two key observations have been made that indicate that the functional significance of compartmentalization can be assessed using HSV-1 infection. First, all of the HATs and HDACs that have been examined are recruited to viral compartments. Second, despite the almost complete recruitment of these enzymes at steady-state, the viral compartments, like transcription factor foci and speckle domains, are dynamic. Thus, the HATs and HDACs are capable of moving into, out of, and between compartments. Interestingly, cells with large viral compartments show decreased levels of highly acetylated histone species (Fig. 8). These results suggest that the study of the influence of HSV-1 infection and progression on the dynamic histone acetylation process will allow a functional determination of the role of nuclear compartmentalization in regulation.

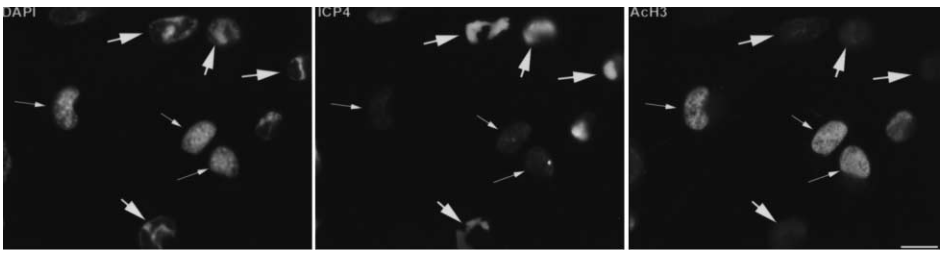

Fig. 8. HSV-1 infected cells show reduced levels of highly acetylated histones. Indian muntjac fibroblasts were infected with herpes simplex virus-1 and cultured for 4 h post-infection. Cells were then fixed and costained with an antibody recognizing a viral protein recruited to the viral replication compartment (ICP4) and an antibody recognizing highly acetylated species of histone H3 (AcH3). The large arrows indicate the positions of cells with well-established replication compartments and the small arrows indicate the positions of cells with very small or no visible replication compartments. The scale bar represents 10 μm.

13. Conclusions

It is very clear that the cell nucleus is organized into many distinct types of compartments. Transcription factors predominantly localize to focal domains that are numerous and found throughout the interior of the cell nucleus. Very recent studies on ligand-induced redistribution of steroid hormone receptors indicate that these compartments are involved in the regulation steroid hormone receptor-mediated activation. Despite this correlation, it is unlikely that these foci reflect direct association with chromatin.

Transcription factor foci compartments are dynamic, with transcription factors continually exchanging into and out of these domains. Two functions for these domains have been proposed; to facilitate the assembly of multiprotein complexes and/or to regulate nucleoplasmic concentrations of specific factors. The identification of specific targeting signals within individual proteins may enable the testing of the role of foci in facilitating the formation of specific multiprotein complexes. Although it is self-evident that association of transcription factors with foci will reduce the nucleoplasmic concentration of individual transcriptional regulators, whether or not this is functionally significant is more difficult to assess. Using HSV-1 infection to study the relationship between recruitment away from the interchromatin space to viral compartments and the dynamics of the histone acetylation process is a promising system for testing the functional significance of regulating nucleoplasmic concentrations of factors.

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