

## Minireview

# Nuclear functions in space and time: Gene expression in a dynamic, constrained environment

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**Abstract** All eukaryotic cells enclose their genome within a dedicated, membrane-bound organelle termed the nucleus, which functions to partition gene transcription from sites of protein translation in the cytoplasm. Despite a great deal of research effort, basic questions about chromosome structure and gene expression mechanisms remain to be answered, including the relationship between the spatial organization of the genome and the transcription machinery. Powerful *in vivo* approaches are allowing researchers to test established *in vitro* concepts within the dynamic cellular environment, while genome-wide screens have enabled rapid high throughput analyses of both structural and functional parameters. In several cases, as highlighted here, this has turned up surprising results and has forced a re-evaluation of models for nuclear structure and gene regulation.

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## 1. Nuclear organization: setting the stage

Within the nucleus, DNA is highly packaged through its association with histone proteins to form nucleosomes, which in turn are further compacted into higher order structures (see Fig. 1A). The complex of DNA with histones and other associated proteins is termed “chromatin”. The formation of chromatin is important both for controlling the compaction of chromosomes and for regulating access to specific DNA sequences as required for both gene transcription and DNA replication (for review, see [1]).

Although the most dramatic change in the condensation state of chromatin occurs at the onset of mitosis, when all chromosomes become highly condensed to facilitate accurate segregation to daughter cells, there are distinct regional variations in condensation within chromosomes during interphase. The most highly condensed regions of interphase chromatin, termed “heterochromatin”, are often found clustered at the nuclear periphery and surrounding the nucleolus (see

Fig. 1B). Heterochromatin generally includes regions in which little or no gene expression occurs and where the DNA is replicated at the late stages of S phase. Less condensed regions, found more towards the interior of the nucleus where the bulk of active transcription sites localize, are referred to as “euchromatin” regions. However, these definitions are not absolute. Certain gene loci, either located within, or targeted to, heterochromatin regions have been shown to actively transcribe, while, conversely, internalized loci within euchromatin regions can be silenced. A great deal of work on budding yeast has focused on the activation and repression of loci at the nuclear periphery, and there is a growing body of evidence that some active genes, such as hexokinase isoenzyme 1 (HXK1), are directly coupled to and regulated by proteins involved in the nuclear pore complex (for review, see [2,3]).

Two recent studies addressed this issue in mammalian cells by targeting inducible gene arrays to the nuclear periphery. Both demonstrated that repositioning was dependent on breakdown and reformation of the nuclear envelope during mitosis. However, the observed effects on transcription differed, with one group showing repression [4] and the other showing that retargeting to the periphery did not prevent transcriptional activation [5]. These apparently conflicting results could reflect gene-specific differences in transcriptional control. Relocalization of two different human chromosomes to the nuclear periphery was also shown to reduce expression of some endogenous genes located near the lac operator site as well as some genes further away from lac operator, while expression of many other genes near the tethering site showed little or no reduction in expression [6]. The significance of regions that tether loci to the nuclear periphery thus remains unclear, although it seems likely that they function in some way to modulate the expression of certain genes during development and differentiation.

In addition to varying degrees of chromosome condensation throughout the interphase nucleus, it is generally accepted that genes are further organized within chromosome territories. These are discrete regions of the nucleus occupied by specific pairs of homologous chromosomes (see Fig. 1B). Although studied in detail, there is still debate about the organization, significance and spatial distinction of these territories (for review see [7–9]). Initial reports, based mainly on fluorescence in situ hybridization (FISH, see Fig. 2) comparing the relative localization of chromosomes and specific gene loci, suggested that chromosome territories were largely distinct, with little or no intermingling. The recent development of a high

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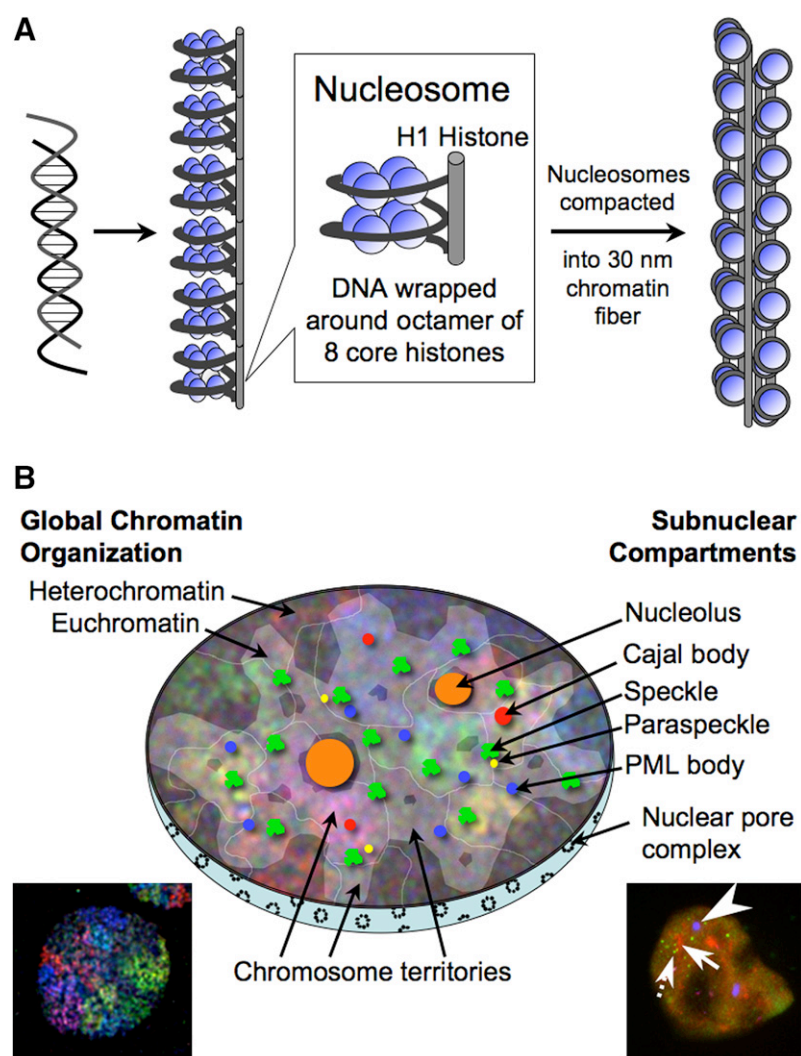


Fig. 1. Spatial organization of the interphase nucleus. Nuclear functions such as DNA replication and transcription are carried out within a highly organized three-dimensional environment. (A) As depicted here, DNA is first wound around a core of histone proteins for packaging into nucleosomes, which are in turn further compacted into chromatin fibers. (B) Packaging of chromatin fibers into higher order structures includes the establishment of open or “euchromatic” regions (light gray), and more condensed, or “heterochromatic” regions (dark gray), often found clustered around nucleoli and at the nuclear periphery. Fluorescent labelling of individual chromosomes (see inset image, bottom left; image courtesy of S. van Koningsbruggen) reveals that they occupy distinct nuclear domains or “chromosome territories”. In addition to chromatin domains, there are other specialized subnuclear compartments that include proteinaceous nuclear bodies (e.g. nucleoli, Cajal bodies, speckles and PML bodies), multiprotein complexes such as transcription factories, and the nuclear pore complexes at the periphery that regulate nucleocytoplasmic transport. The inset image (bottom right) shown co-immunostaining of Cajal bodies (blue, arrowhead), speckles (red, arrow) and paraspeckles (green, hashed arrow) in a fixed HeLa human fibroblast cell.

resolution cryo-FISH technique, however, demonstrated significant intermingling during interphase, suggesting that chromosome territories interact more than previously thought [10]. In addition, the frequency of chromosome translocations was correlated with the degree to which they intermingled.

Genomic instability and chromosome translocations are hallmarks of tumor cells (for review, see [11]), and although defining the mechanism of translocation is important, it does not explain why they occur. Chromosome translocations are, at least in part, a consequence of the non-random spatial chromosome organization observed in the nucleus. Chromosome pairing is not, however, confined to tumorigenic cells. A good example of the functional significance of interchromosomal interactions is the transient homologous pairing involved in X chromosome silencing (for review, see [12]). It has been demonstrated that there must first be a transient physical interac-

tion between the two X chromosomes, after which the decision to inactivate one of them is made [13]. The silenced locus has also been shown to specifically target to the perinuclear region during S phase, and this targeting is important for both silencing and replication [14].

Inheritance of spatial chromatin organization is believed to be important for long-term stability of the genome, and therefore imaging-based studies have also addressed the question of whether global chromosome positions are transmitted through mitosis. Using photobleaching techniques to monitor relative chromosome positions in live cells, initial results were conflicting (for review, see [15]). When gene loci were monitored by time-lapse imaging with respect to both radial position and association with nuclear compartments in cells exiting mitosis, it was observed that spatial organization was not inherited but refined during G1, when significant movement occurs [16].

Similar results were found using photobleaching techniques, where cell cycle stages were monitored and normalized to control for heterogeneity. Global chromosome organization was shown to undergo rearrangement during G1, but then, after establishment, was maintained throughout the rest of the cell cycle [17]. Interestingly, the spatial chromosome reorganization observed in G1 still occurred in the presence of the transcriptional inhibitor 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), suggesting that it is not dependent on active transcription [16].

Chromosomes share the intranuclear space with several types of nuclear bodies, including nucleoli, Cajal bodies, PML bodies, speckles and paraspeckles (Fig. 1; for review, see [18]). These bodies primarily occupy the interchromatin space and in some cases are associated with specific gene loci and/or their RNA products. The nucleolus, for example, assembles at chromosomal loci containing the tandemly repeated rRNA gene clusters and this subnuclear compartment is the site of ribosome subunit biogenesis from ribosomal RNA transcription to rRNA processing and ribosome subunit export [19]. Although the nucleolus is a defined structure, most of its protein and RNA–protein (RNP) constituents are dynamic and in constant flux. Similar results have been found for most other nuclear bodies. This has led to the proposal of a “self-organization” model, in which distinct nuclear sub-compartments, such as the nucleolus, represent the collective sum of protein interactions occurring at that particular site (for review, see [20,21]). This dynamic protein turnover also allows for rapid changes in the composition of nuclear bodies in response to cellular perturbations, and mass spectrometry-based studies are now able to characterize these “dynamic proteomes” and explore their functional significance [22,23].

Cajal bodies are also found associated with specific gene loci, including histone and U snRNA genes (for review, see [24]), and Cajal body integrity has been shown to depend on active U snRNP biogenesis [25]. When the levels of certain proteins that inhibit either U snRNP maturation, or export, are knocked down, Cajal bodies are lost and the protein p80 coilin disperses into small foci containing snoRNPs. This suggests that Cajal bodies, like the nucleolus, have distinct subdomains that may preferentially process either U snRNPs or snoRNPs.

Unlike nucleoli, nuclear speckles, which correspond to interchromatin granule clusters, do not appear to assemble only at specific gene loci, but instead are found in proximity to sites of active transcription. Speckles are enriched in pre-messenger RNA splicing factors. However, they may not correspond to active sites of splicing (for review, see [26]). For example, they are enlarged and accumulate in the absence of transcription when splicing levels are expected to be low.

Paraspeckles, so named because they are normally found adjacent to speckles [27], are not enriched in pre-mRNA splicing factors but instead contain the protein complex PSP1-p54nrb, which has been shown to localize to paraspeckles in an RNA dependent manner. Paraspeckles normally disperse as cells enter mitosis and then re-form in G1. If reinitiation of transcription at the end of mitosis is blocked using the inhibitor DRB, paraspeckles do not reform. When DRB is removed, transcription resumes and paraspeckles assemble [28]. Interestingly, paraspeckles may play a role in ensuring the nuclear retention of specific forms of RNA. Evidence for this comes from a study in which paraspeckles were shown to be a site of retention for CTN-RNA, which is released during

stress conditions and cleaved to form the protein-coding RNA mCAT [29].

PML nuclear bodies have not been linked to any specific gene loci and RNA does not accumulate within them, but they have been shown to associate with gene dense, transcriptionally active chromatin regions (for review, see [30]). PML bodies can be disrupted by RNAi-based knock down of the PML protein, but this had no major effect on the expression of certain genes shown statistically to be closely associated with PML bodies, suggesting that although the bodies form in nuclear compartments containing high transcriptional activity, they may not directly regulate transcription of genes within this region [31]. Recently, however, an elegant experiment in which transcription reporter plasmids were targeted to PML bodies demonstrated a promoter-specific regulation of transcription occurring within the PML body microenvironment [32]. Clearly, further work needs to be carried out to determine both the structural and functional relationships between chromosomes and subnuclear domains.

## 2. Large-scale analyses of genome organization

Although FISH is not a high throughput method, the development of combinatorial multicoloured FISH has increased the number of genes that can be monitored within the same nucleus. These techniques are now being employed both to study genome organization and as diagnostic tools for detecting chromosome abnormalities. For example, Levsky et al. [33], recently assessed the spatial organization of 10 active genes, mostly on different chromosomes, and observed that sites of gene expression were mainly excluded from the nuclear periphery. No apparent clustering at common sites of transcription was observed, however, which may not be surprising given that the chosen genes were not known to be co-regulated. Sharing a transcription site may depend on either close proximity of the two loci prior to activation, or on the coincident looping out of distinct chromosome regions into the same area.

In contrast to the visual assessment of gene proximity offered by FISH approaches, chromosome conformation capture (3C; Fig. 2) was developed to identify physical interactions between gene loci by converting them into ligation products (for review, see [34]). These ligation products can be analyzed by PCR to determine both the presence and the frequency of a specific interaction. Although originally limited to the specific screening of either known or candidate interactions, it has recently been extended by the development of 4C (3C on Chip) and 5C (3C carbon copy) techniques. These methods use differing approaches to amplify the original 3C library and permit genome-wide screening, either on dedicated microarray libraries, or via high-throughput sequencing (e.g. 454 or Solexa/Illumina).

The 4C approach was recently used to perform an unbiased, genome-wide search for DNA loci that contact either an active  $\beta$ -globin locus on chromosome 7 in fetal mouse liver, or the inactive locus in fetal brain [35]. Potential hits were validated by high resolution cryo-FISH. Both the active and inactive genes displayed interchromosomal (cis) contacts, but were also shown to be engaged in several long-range intrachromosomal (trans) interactions. The active locus preferentially contacted transcribed loci elsewhere on chromosome 7, supporting the idea that chromosomes fold into areas of active chromatin,

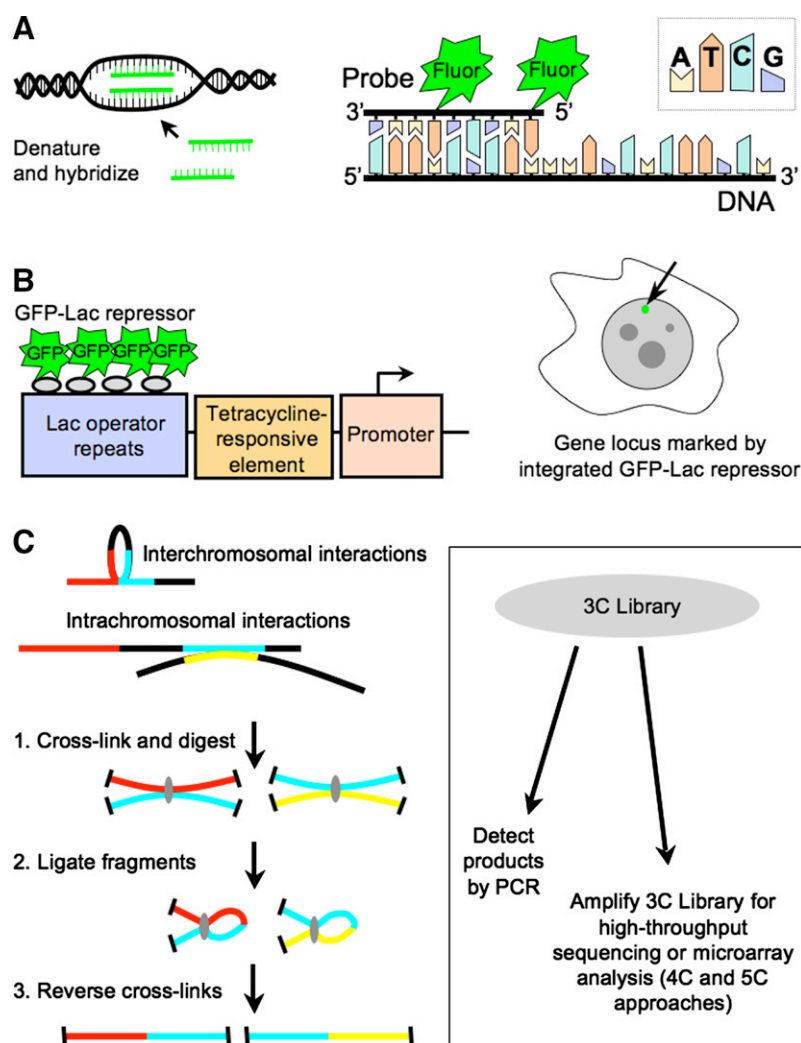


Fig. 2. Mapping 3D genomic organization. (A) DNA FISH is an effective method for labelling specific gene loci. Fluorophore-tagged probes are hybridized to denatured genomic DNA in fixed cells and detected by fluorescence imaging. (B) The lac operon/FP-lac repressor system is widely-used for imaging gene loci in live cells, by random or targeted incorporation of a transgene that includes multiple repeats of the lac operator. Co-expression of fluorophore-tagged lac repressor protein reveals the position of the locus within the cell (arrow). (C) Chromosome conformation capture (3C) is a powerful technique for identifying and mapping both interchromosomal and intrachromosomal interactions, by converting physical interactions into specific ligation products. Initially limited to testing specific interactions by PCR analysis, recent advances (4C and 5C approaches) combine 3C with amplification steps to allow genome-wide non-biased screening utilizing high-throughput methods such as microarrays and 454 or Solexa/Illumina sequencing.

while the inactive locus contacted different, transcriptionally silent genes.

Development of the 5C approach enabled mapping of both cis- and trans-interactions of the human  $\beta$ -globin locus [36]. By comparing interactions from a 400 kb region containing this locus to a 100 kb conserved region of low gene density, this study validated looping interactions within the locus previously identified by 3C and RNA-TRAP techniques, and identified a new interaction between the  $\beta$ -globin locus control region and the  $\gamma$ - $\delta$ -globin intergenic region. Intriguingly, this region has been implicated in the control of globin gene switching during development [37].

### 3. Chromatin modifications and DNA–protein interactions

Global and localized changes in chromatin structure are driven, at least in part, by post-translational modifications,

including methylation and acetylation of specific histone residues. Modification of the DNA itself, such as base methylation of cytosine residues, is also important and involved in the epigenetic marking of gene loci. Other global chromatin studies are thus addressing the significance and mechanism of complex post-translational modifications, including ADP-ribosylation, ubiquitination, phosphorylation, acetylation and methylation, that occur on both DNA and DNA-associated proteins as transcriptional regulators and epigenetic markers (for review, see [38,39]). Quantitative proteomic analyses of multisite modifications is now within reach, with a recent study mapping acetylation and other modifications on histones to quantitatively study the large-scale affect of histone deacetylase inhibitors in a site-specific and dose-dependent manner [40]. Genome-wide profiling of histone methylations has also been carried out using a combination of ChIP and high-throughput Solexa/Illumina sequencing [41]. In this study the authors started with a mononucleosome template and carried out a

series of ChIP experiments using antibodies specific for 20 histone lysine and arginine methylations, the variant histone H2A.Z, RNA polymerase II and CTCF. By comparing the results to 12726 human genes of which expression levels in resting cells are known, they found typical histone modification patterns at promoter, insulator, enhancer and transcribed regions, and also showed links to gene activation and repression.

Another method for mapping protein–DNA interactions is DamID, in which bacterial DNA methyltransferase is tethered to the protein of interest, thus targeting it to specific sites where it methylates adenosine residues (which does not occur naturally in eukaryotes [42]). When combined with cDNA array detection, it allows mapping of *in vivo* binding sites of chromatin-associated proteins. Previously used to map sites associated with Lamin B [43], the same group more recently used it to define heterochromatin, by probing interactions between HP1 and other chromatin components [44]. In brief, the sequence fragments (between two methylated GATC regions) generated by targeted HP1-Dam fusions were amplified and identified by microarray analysis, and compared to those generated by a Dam alone control. Despite the widely-held belief that heterochromatin regions are repressive, HP1 was most frequently associated with active genes.

Large-scale proteomic analysis of DNA-associated protein complexes is also being used to define active genes and the mechanisms of their activation/repression. Chromatin immunoprecipitation (ChIP) techniques have helped to localize proteins to specific genes and, when combined with microarray analysis in a “ChIP-on-chip” approach, can identify target sites for a particular protein on a genome-wide level. ChIP can also be combined with MS-based proteomics to immunoprecipitate a tagged protein of interest and identify both where it binds on the DNA and what other proteins are associated with the complex (for review, see [45]). A ChIP-seq approach [46] combining ChIP with high-throughput Solexa/Illumina sequencing was recently used for genome-wide mapping of *in vivo* protein–DNA interactions. In this initial study, a well-defined chromatin binding protein (NRSF, a.k.a. REST) was used to demonstrate the power of the technique, by confirming the large number of binding sites previously identified by ChIP-QPCR. Short sequence reads were mapped onto the known genome and factor binding sites mapped with  $\pm 50$  bp accuracy. Importantly, unlike microarray approaches, in which large plasmid libraries must be constructed (and you can only find what you are looking for), this technique sequences every interaction site.

The ChIP-Seq approach has also been used to generate genome-wide maps of nucleosome positioning in human CD4+ T cells under both resting and activated conditions, by direct high-throughput sequencing of nucleosome ends [47]. This powerful approach provides a comprehensive view of the transcriptional landscape, from the smaller-scale binding of specific protein factors to DNA to the larger-scale organization of nucleosome structure.

#### 4. Diffusional and long-range chromatin motion

Direct evidence for chromatin motion has been provided primarily by experiments utilizing the lac operon system, in which transgenes containing multiple copies of the lac operator (see

Fig. 2B) are transiently transfected or stably incorporated into cells and then monitored by co-expressing FP-tagged lac repressor (for review, see [48]). Early experiments in yeast demonstrated constrained diffusional motion for labelled loci, finding no indication of active or motor-driven transport [49]. When loci at different positions were compared, namely those within the interior vs. those at the periphery, the internal loci were found to be more dynamic [50]. Random incorporation of short (128-mer) lacO repeats into mammalian cells demonstrated a similar relationship between chromatin movement and nuclear localization, with constrained diffusion measured for those loci found in close proximity to the nuclear periphery and nucleoli [51]. Recently, constrained diffusion was also demonstrated for chromatin located near nuclear speckles, using photoactivatable GFP-histone H4 [52].

Although general chromatin mobility appears to be minimal during interphase, the spatial rearrangements observed following gene activation and during differentiation point to long-range movements of specific loci, and the mechanisms involved are still not explained. It remains unclear, for example, whether long-range movement of chromosomal loci is direct, or if it is the indirect effect of chromatin ‘looping out’, while the chromosome itself maintains a fixed position within the nucleus. It is also unclear whether or not it is energy-dependent or governed by some type of motor system (for review, see [53]).

In an attempt to address these questions, localization studies have been carried out by time-lapse imaging in live cells. Long-range movement from the periphery to the interior of the nucleus was observed for a lacO/FP-lac repressor tagged gene locus within 1–2 h of activation [54]. This movement was not dependent on transcription. Overexpressing mutant forms of the motor proteins actin and myosin did inhibit locus movement, however, suggesting the possible involvement of some type of motor system. Both actin and myosin are found in the nucleus, and nuclear forms of myosin have been found in the RNA polymerase II complex and at transcription factories [55]; for review, see [56]. Nuclear motor proteins have also been implicated in the nuclear rearrangements observed in estrogen receptor  $\alpha$ -enhanced transcription [57].

Long-range movement of gene loci was also observed recently for the U2 snRNA gene locus, which was already known to associate with Cajal bodies [58]. Both RNA and DNA FISH, in combination with a GFP-tagged Cajal body marker, were used to demonstrate that approximately 6–7 h after induction of a stably integrated U2 snRNA array, the locus moved from within the interior of the chromosome in which it was integrated to a nearby Cajal body (see Fig. 3). Live cell imaging of both the locus and Cajal bodies demonstrated that the locus moved to the Cajal body, which remained relatively immobile. Overexpression of an actin mutant disrupted movement, again suggesting that some sort of motor system may be involved in long-range movement of gene loci, although the mechanism involved is unclear.

#### 5. Transcription factories and the transcriptional landscape

A high resolution cryo-FISH study revealed significant intermingling of what were previously thought to be spatially distinct chromosome territories and also demonstrated the

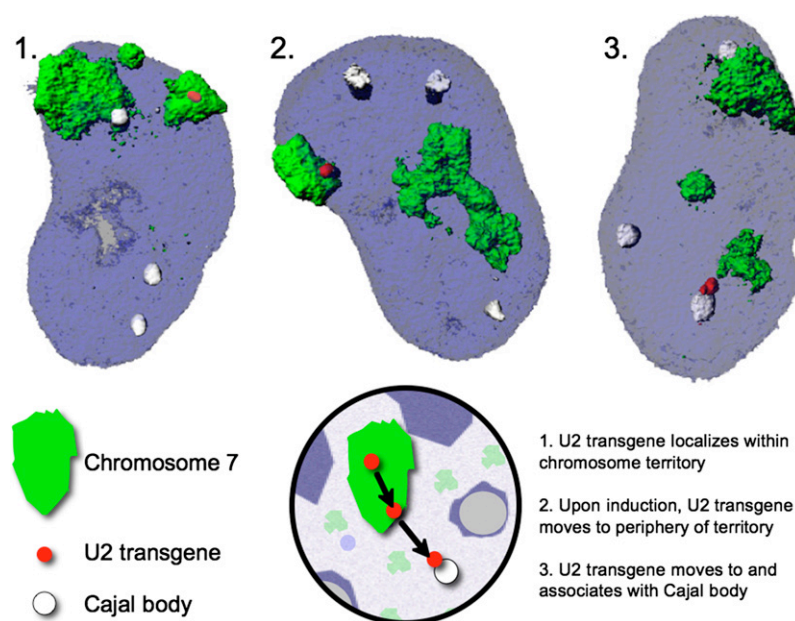


Fig. 3. Long-range movement of a gene locus. Isosurface views of individual cells expressing the U2 transgene (red). Cells were counterstained to visualize DNA (purple), chromosome 7 (green) and Cajal bodies (white). When induced, the transgene, initially found within chromosome 7, first moves to the periphery of the territory and then to a Cajal body. Image courtesy of A.G. Matera and M. Dundr.

presence of transcription factors in these regions of intermingling [10]. This supports the idea that at least some chromatin associations are transcription-dependent, i.e. several loci targeted to a shared site of transcription. This concept of colocalization of genes from different loci in shared sites of transcription was proposed several years ago and termed “transcription factories” (for review, see [59]).

Transcription factories are observed as multiple discrete foci spread throughout the nucleoplasm when transcripts are visualized by pulsed incorporation of labelled nucleotides. Estimates of the number of foci in eukaryotic nuclei range from 500 to 10000. Because the number of transcription sites is always less than the number of active RNA polymerases, the model proposes that several polymerases and associated factors share a common transcription factory. In addition, several lines of evidence point to the idea that the transcription factory remains immobilized while the DNA template moves along it, extruding recently transcribed mRNA. This process was recently visualized using a combination of FISH with a tandem gene array tagged with GFP to probe the organization of chromatin at a transcription site. A cloud of decondensed, recently transcribed chromatin was shown to border transcription sites marked by either BrUTP incorporation or Pol II staining [60]. A related concept of a “replication factory”, in which sister replication forks generated from the same origin remain associated within an immobilized functional complex while the DNA moves through it was recently proposed [61], suggesting that this is a common mechanism for events based on the DNA template.

A dynamic relocalization of active genes to transcription factories was demonstrated by Osborne et al. [62] by comparing positions of loci in either the “on” or “off” state. Correlations in spatial association were observed specifically when genes were active, suggesting the presence of transcription factories. This work was recently extended for a specific locus that includes the *c-myc* gene, which relocated to a transcription fac-

tory occupied by the *Igh* gene locus upon activation [63]. Interestingly, the *c-myc* and *Igh* loci are found on chromosomes 15 and 12, respectively, and are frequent translocation partners in disease. Comparison of an active and inactive allele of the same gene within the same nucleus [64] showed that they occupied different radial positions, again supporting the idea that the position of a gene does not determine its activity, but rather is a consequence of its activation state.

Not all genes are expressed in any given cell type, and yet recent studies have demonstrated that a surprising number of them are poised and ready to express. For example, in a study assaying the effect of heat shock on transcription in yeast, a large number of promoters were seen to assemble into partial pre-initiation complexes prior to heat shock. Upon heat shock, sites of active transcription recruited polymerase and evicted the H2A.Z nucleosomes, suggesting that the assembly of pre-initiation complexes is a way to prepare for stress [65]. Genome-wide analysis of promoter regions in human ES cells revealed that ~74% of them had markers indicating transcriptional initiation [66]. These markers included RNA polymerase II and specific histone modifications such as H3K4me3, H3K9 and 14Ac (for review, see [39]). This suggests that the majority of promoters initiate transcription and are then regulated downstream of this step, either by rapid degradation of transcripts or regulation at the elongation step (for review, see [67]). A recent whole genome tiling array performed on human ES cells to determine their transcriptional landscape showed a similar global hyperactivity, suggesting that this may be a hallmark of their plasticity [68]. With most genes poised and ready to activate, it is possible that specific loci are then silenced as the cells differentiate.

Global transcriptional activity is not necessarily limited to stem cells. When primary hepatocytes and B lymphocytes were profiled, H3K4me3 marker was found at the majority of promoters for both active and repressed genes, and the initiating form of RNA polymerase II was found at ~40% of them

[66]. This could indicate a general phenomenon of transcriptional initiation at most genes, which would be an efficient means of responding quickly to physiological changes.

## 6. Monitoring gene expression in vivo

Having set up the nucleus as a dynamic 3D structure containing a large volume of condensed yet mobile chromatin and subnuclear organelles maintaining a constant flux of proteins and RNA, it is only to be expected that gene expression itself can also be a dynamic event. Protein–chromatin interactions are transient, enabling fast scanning of the genome by transcription factors and leaving binding sites constantly available for scanning by other transcription factors (for review, see [20]). Low expression of a fluorescent lac repressor fusion protein in *Escherichia coli* enabled direct visualization of a single repressor, allowing quantitation of the search time for it to scan and find its binding site on DNA. Using this approach, transcription factor dynamics were monitored at the single-molecule level. Addition of IPTG resulted in rapid dissociation of the repressor from the binding sites, and upon removal of IPTG, repressor could rebind to its operator sites within 1 minute. Further analysis by single-molecule tracking on flow-stretched DNA in vitro led to an estimated residence time for repressors searching for an operator on the order of 5 ms [69].

A surprisingly dynamic turnover has also been observed in mammalian cells for the transcriptional activator NFκB, which has a high affinity for DNA and is thought to help form stable “enhanceosomes” on specific sites dispersed throughout the genome (for review, see [70]). Although quite stable in vitro, when analyzed in vivo using GFP-NFκB, protein turnover is observed within 30 s [71]. This suggests that although the enhanceosome nuclear body is stable, like other nuclear bodies its components exchange rapidly. The authors propose a model in which the residence time of NFκB on specific binding sites defines a stochastic window during which general transcription factors and other required components must collide with the same regulatory region for transcription to occur. Such rapid exchange would also permit constant fine tuning of the components of the enhanceosome, and enable a quick response to environmental changes (e.g. if the stimulus that activated them was withdrawn).

It is not only the gene locus and/or associated proteins that can be visualized in live cells by fluorescent reporter molecules. The RNA products themselves can also be monitored in real time, either by labelling with fluorescent probes or utilizing the MS2–FP system, which is based on incorporation of an RNA stem loop that binds with high affinity to a co-expressed FP-tagged MS2 coat protein (see Fig. 4A and B). Both methods have been used to demonstrate that newly transcribed RNA moves away from sites of transcription in all directions, by free diffusion [72,73].

MS2–FP was recently used to monitor the time course of gene transcription, by stable integration of a cassette of MS2 stem-loops into a single endogenous developmental gene in *Dictyostelium* [74]. Rather than a continuous signal, pulses of transcription were observed (see Fig. 4C). The pulses were irregular in both length and spacing, with a mean on/off time of 5–6 min. Transcriptional pulsing or “bursting” has also

been demonstrated in bacteria using an MS2–FP system and a steady input of inducer [75,76]. Just as with dynamic protein constituents of nuclear bodies or protein complexes, a potential benefit of pulsing over continuous transcription would be greater sensitivity and a more rapid response to environmental changes.

The MS2–FP system has also been combined with the lac operator/FP-lac repressor system and an FP-tagged protein product in an inducible transgene for real time imaging of transcription in single cells. The gene locus is marked with lac operator repeats (visualized by co-expressing CFP-lac repressor), while RNA transcribed from the array contains MS2 repeats in its 3′-untranslated region and can be detected through the binding of MS2–YFP. The array comprises 200 copies of the transgene, which also encodes CFP fused with a peroxisome-targeting peptide, to provide convenient detection of the translated protein in the cytoplasm. Gene expression can thus be followed from gene induction to RNA transcription and protein translation. This powerful approach enables changes in chromatin structure to be correlated with the progression of transcriptional activation, and the relative timespan for each step calculated [77].

The system has also been adapted to perform single cell kinetic measurements of promoter binding, initiation and elongation in single cells [78]. Using a combination of photobleaching and photoactivation coupled with mathematical modelling, single-cell kinetic measurements were made of promoter binding, initiation and elongation events. Transcription was inefficient, with only ~1% of polymerase–gene interaction events resulting in productive synthesis of full length mRNA. The authors also measured a faster rate of elongation than previously detected (~4.3 kb/min) and obtained evidence that a subset of polymerases pause during transcription for extended periods. This study illustrates what will become possible in terms of single cell analysis of gene expression as more gene templates are analyzed and as further improvement in the sensitivity of detection of fluorescent signals allows characterization of smaller arrays or even single copy genes.

The MS2–FP system was also combined with photobleaching experiments to analyze the dynamics of specific RNAs [79]. Because there is a concern that the binding affinity of the probe to target RNA can influence the diffusion estimates of the complex [80], an MS2 variant was designed that has a very high affinity for the stem loop. When incorporated into an RNA that stably associates with nucleoli (U3), photobleaching experiments showed little dissociation within 10 min, indicating that the MS2–FP stays bound to RNA over the time course of a typical transcription experiment. HIV-1 was used as the experimental system because it is integrated into the genome as a single transcription unit. By including MS2 binding sites in the 3′ untranslated region of the HIV vector, transcription rates could be monitored at a single site by FRAP analyses of RNA-bound MS2–GFP. An elongation rate of 1.9 kb/min was calculated for this system. Interestingly, the polymerases were observed to remain at transcription sites 2.5 min longer than nascent RNAs, with a rapid release of mRNA once polyadenylation was initiated. This supports the idea that polymerases either pause, or else lose processivity, after passing the polyadenylation site. In contrast with previous studies [78,81], transcription was relatively efficient, with most polymerase–gene interactions resulting in productive transcription.

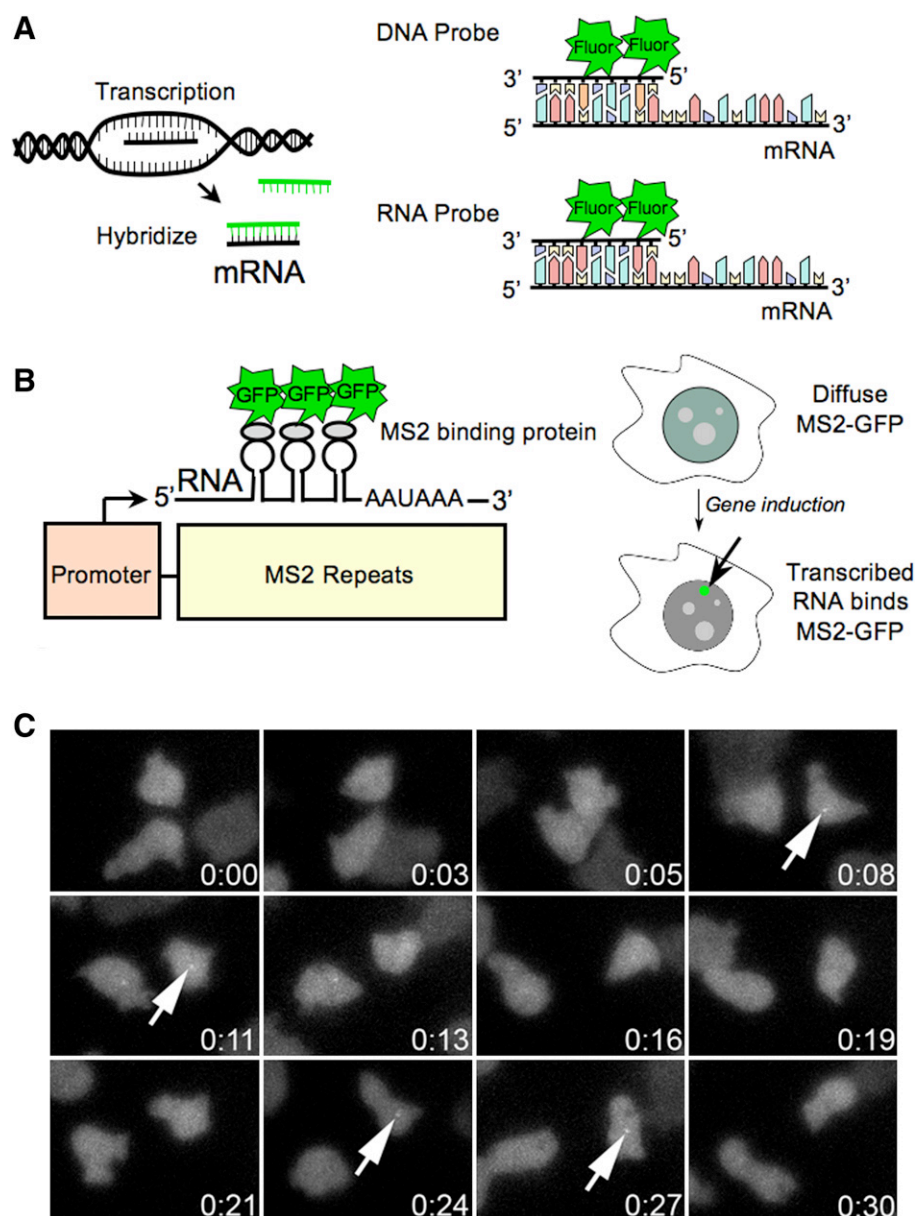


Fig. 4. Visualizing transcription. (A) RNA FISH, in which fluorophore-labelled probes (either DNA or RNA) are used to label fixed cells, reveals sites of transcription of specific mRNA sequences within the nucleus. (B) The MS2–GFP system combines transient expression or stable incorporation of a transgene expressing the phage RNA stem–loop MS2 with expression of GFP fused to the phage MS2 coat protein that binds with high affinity to the MS2 RNA. This system can be used to monitor sites of transcription in live cells (arrow). (C) Analysis of transcription in live cells reveals that it occurs in “bursts” or “pulses” (arrows), with varying times of dormancy in between. Time-lapse images courtesy of J. Chubb.

A caveat to these *in vivo* experiments is that both the use of FP proteins as reporter molecules and the current requirement for multicopy gene arrays to visualize the processes may have an affect on the transcription parameters being measured. Further improvement in the sensitivity of detection of fluorescent signals should facilitate characterization in future of smaller arrays or even single copy genes. In addition, care must be taken when using chemical inhibitors to assess changes in cellular processes, such as the energy and/or transcription-dependence of chromatin and protein/RNA mobility. For example, several laboratories have demonstrated that nuclear stress induced by compounds commonly used to deplete cellular energy, such as sodium azide and 2-deoxyglucose, leads to dramatic chromatin reorganization [72,82,83]. The chromatin

structural changes were demonstrated by monitoring a photo-bleached grid of YFP–H2B [72]. This is important because when the MS2–FP system was used to monitor mRNP movement in live cells, it was apparent that a decrease in temperature did not affect the diffusion rate, confirming that these complexes move by Brownian motion. However, if chemical inhibitors were used to deplete energy, then the mRNPs showed reduced mobility and concentrated in subnuclear regions surrounded by condensed chromatin. Bearing in mind the data showing the effects of inhibitors on global chromatin condensation, a likely explanation for the data is that the space available for free diffusion was decreased and that this affected mobility, rather than a genuine involvement of a motor-based movement mechanism.

With regard to transcriptional inhibition, the response to it is complex and dependent on the inhibitor employed. When the dynamics of FP-tagged RNA polymerase II were analyzed by FRAP in the presence of various inhibitors, some elicited the expected response, such as the decrease in the engaged pool of polymerase observed when the cyclin-dependent kinases that phosphorylate and activate it were inhibited (e.g. by treatment with DRB). However, two separate effectors that both cause stalling of the polymerase in vitro, i.e., cisplatin and UVC irradiation, should both lead to an increase in the engaged fraction, and yet they show opposite effects [84].

## 7. Conclusion

Much of our current picture of the cell nucleus has derived from methods that analyze statistical averages of cell populations. While important, this may be misleading in terms of what is taking place at the single cell level. For example, heterogeneity in gene expression between cells was demonstrated by a study in which the response of gene loci to serum stimulation was monitored [85]. Protein blot analysis (a typical “population average” ensemble approach) showed that each gene studied was activated 3–4-fold. At the single cell level, however, some loci were not activated at all and others activated at a higher level. The transcriptional pulsing observed in live cells [74] would also have been averaged out in population-based studies, demonstrating the importance not only of single cell measurements, but also of live cell measurements.

Advances in imaging, MS-based proteomics and deep sequencing technologies have all contributed to the recent progress in analyzing the dynamic events associated with nuclear structure and function, particularly with respect to chromatin organization and transcriptional regulation. This includes the development of fluorescent reporter systems to monitor gene transcription over time in live cells, high throughput methods to rapidly identify chromatin–chromatin and chromatin–protein interactions and quantitative proteomics methods that facilitate detailed mapping of complex modifications that occur on chromatin-associated proteins.

As detailed here, these new approaches have already thrown up a few surprises, including the dynamic association of transcription factors with DNA and the long-range movement of gene loci within the nucleus, possibly driven in some cases by motor proteins. So long as the sensitivity and resolution of imaging, proteomics and DNA sequencing techniques continue to improve, it is feasible that all of the nuclear events discussed here will one day be analyzed for endogenous genes at the level of the single cell and this is clearly the major goal for developing a detailed understanding of how gene regulation mechanisms actually occur in vivo.

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