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Seeing is Believing: Visualizing Transcriptional Dynamics in Single Cells

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Abstract

For a gene to be expressed, the functions of multiple molecular machines must be coordinated at the site of transcription. To understand the role of nuclear organization in transcription, it is necessary to visualize the dynamic interactions of regulatory factors with chromatin and RNA. It is currently possible to localize individual transcription sites in single living mammalian cells by engineering reporter gene constructs to include sequence elements which permit the visualization of nucleic acids *in vivo*. Upon stable integration, these transgenes form chromatinized arrays, which can be imaged during activation to obtain high-resolution quantitative information about transcriptional dynamics. Modeling can suggest new hypotheses about gene regulation, which can be tested both in the single-cell imaging system and at endogenous genes. This gene-specific imaging strategy has the potential to reveal regulatory mechanisms, which would be difficult to imagine outside of single living cells.

Keywords

Transcription; Chromatin; Single-cell imaging; Nuclear organization; Histone H3.3

Presentation

Genes are activated when transcription factors interact with specific DNA sequence elements to initiate a cascade of events, including chromatin remodeling, transcription and RNA processing (Coulon et al., 2013). Although these steps are often studied as distinct mechanisms, they are, in fact, highly interdependent (Hager et al., 2009). To achieve an integrated view of gene expression, it is necessary to understand how they are coordinated at transcription sites. By combining advances in live-cell imaging and auto-fluorescent protein technology with reporter gene engineering, it is possible to visualize gene activation at specific transcription sites in single cells. The information provided by this experimental approach about the timing and spatial organization of gene expression can lead to the discovery of novel regulatory mechanisms. This review describes the history and fundamental components of this gene imaging strategy, as well as its limitations and future applications.

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Transcription and Chromatin: A Mutualistic Relationship

In cells, the genome is packaged into an assemblage of DNA and proteins called chromatin. Chromatin is the interface through which the transcription machinery gains access to the gene template. Therefore, chromatin is a critical regulator of transcription, and conversely, transcription drives changes in chromatin organization. Most conventional techniques for studying gene regulation (e.g. chromatin immunoprecipitation, RT-PCR, proteomics, etc.) require the disruption of cellular architecture rendering them unable to reveal how factors are temporally and spatially organized at genes during activation. Visualizing factor dynamics at individual transcription sites in single living cells, however, can provide insight into gene-specific regulatory mechanisms as well as genome organization and epigenetic inheritance because of the important role transcription plays in these processes (Denholtz and Plath, 2012; Misteli, 2010).

The Nucleus: A Dynamic Network of Functional Interactions

In addition to its effects on chromatin, transcription also significantly impacts nuclear organization (Bickmore and van Steensel, 2013; Ritland Politz *et al.*, 2013). In the nucleus, factors with similar functions often co-localize in discreet membraneless structures called nuclear bodies (NBs) (Dundr and Misteli, 2010; Mao et al., 2011b) [e.g. PML nuclear bodies (PML-NBs), Cajal bodies, nuclear speckles, nucleoli, etc.]. Although the functions of most NBs are incompletely understood, alterations in their organization correlate with changes in gene expression in several diseases indicating that their structures are linked to functions. For example, the oncogenic fusion protein, PML/RARA (retinoic acid receptor α), which causes acute promyelocytic leukemia (APL), both represses genes required for myeloid differentiation and increases PML-NB numbers (de The *et al.*, 2012). During herpes simplex virus-1 (HSV-1) infection, ICP0, the E3 ubiquitin ligase required to repress innate immunity and reverse viral latency, dissolves PML-NBs and alters host and viral gene expression (Boutell and Everett, 2013).

Initially, NBs were thought to be stably assembled structures because they appear to be highly organized in electron micrographs and immunofluorescence images (Rouquette *et al.*, 2010). However, these techniques require fixation, and therefore, can only provide “snapshots” of cells at single time points. When auto-fluorescent proteins [e.g. green fluorescent protein (GFP)] were used to examine protein movement in living cells, they revealed that NBs are actually highly dynamic (Phair and Misteli, 2001). Although at steady state, their GFP-tagged components appear static, the rapid recovery of fluorescent signal after photobleaching suggests that they are continuously associating and dissociating (Misteli, 2008a). These studies indicate that the nucleus is simultaneously organized and dynamic, which is consistent with the ability of cells to rapidly respond to stimuli (e.g. growth factors, heat shock, viral infection, etc.) and efficiently execute regulatory events (e.g. DNA repair, transcription, DNA synthesis, etc.) (Misteli, 2008b).

Connecting NBs with Molecular Functions

Among NBs, the structure/function relationship is best defined for nucleoli, sites of RNA pol I transcription, pre-rRNA processing, and ribosome assembly (Hernandez-Verdun, 2006; Raska et al., 2006). The repetitiveness of ribosomal DNA (rDNA) provides the resolution

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needed to easily identify these RNA pol I transcription sites in single cells. The high concentration of substrate within nucleoli makes it possible to discern sub-domains, which likely function to accelerate enzymatic and assembly reactions (Boulon et al., 2010; Pederson, 2011). It is thought that nuclear factors self-organize around gene expression and genome regulatory events and that the structural integrity of NBs and their subdomains is defined by transient protein–protein and protein–nucleic acid interactions (Dundr, 2012; Dundr and Misteli, 2010).

In contrast to the multi-copy rDNA which comprises RNA pol I transcription sites, it is technically difficult at this time to visualize single endogenous RNA pol II genes in single living cells because of detection limitations. However, multi-copy arrays of transgenes containing sequence elements that allow nuclei acids to be visualized *in vivo* can be easily localized (Rafalska-Metcalf and Janicki, 2007; Yunger et al., 2013a). When RNA pol II transcription units are included in these transgenes, transcriptional dynamics can be directly visualized at these arrays. This approach has also been used to link NB biogenesis to transcriptional mechanisms (e.g. paraspeckles, histone locus bodies with associated Cajal bodies**,** and PML-NBs) (Mao et al., 2011a; Newhart et al., 2012; Shevtsov and Dundr, 2011). Therefore, in addition to transcriptional dynamics, this methodology can be used to study nuclear organization.

Assembling the “Visible Gene”

Synthetic reporter genes are routinely constructed to decipher the functions of genetic elements in transcription. These reporter gene assays are quantitative because measurable proteins (e.g. GFP, luciferase and β-galactosidase) are encoded in the transcription units. However, in its current usage, this methodology has a number of significant limitations. If cellular fixation and/or rupture are required for protein detection, it precludes analysis of the same sample over time resulting in limited and/or arbitrary time point selection. Assays which average effects in cell populations obscure transitory events and cell-to-cell variability. When reporter constructs are transiently expressed, transcription is measured from non-chromatinized templates. Additionally, protein-based gene activity assays are several degrees removed from events at the transcription site, and thus, provide only limited insight into *cis*-acting regulatory mechanisms.

A pivotal advance in our ability to directly study transcription in single cells came from the use of protein/nucleic acid interaction units from bacteria and bacteriophage to visualize DNA (e.g. the lac repressor/operator) and RNA (e.g. MS2 coat protein and *Pseudomonas aeruginosa* PP7 bacteriophage/hairpin sequences) in living cells (Darzacq et al., 2009; Hocine et al., 2013; Rafalska-Metcalf and Janicki, 2007; Wu et al., 2011). When synthetic reporter genes, containing repeats of these elements, are stably expressed, they integrate into single genomic sites as chromatinized multi-copy arrays (Rafalska-Metcalf and Janicki, 2013). Figure 1 shows the linear structure of this type of transgene and the cellular localization patterns of the proteins used to track gene expression. Lac repressor, fused to an auto-fluorescent protein, makes it possible to visualize the “transcription site” chromatin in both the inactive (condensed) and activated (decondensed) states (Figure 1). Similarly, the MS2 coat protein, which interacts with the stem loop structure of the translational operator

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as a dimer, permits visualization of the transcribed RNA at both the transcription site and throughout the nucleus (Figure 1). The mRNA encodes Cyan Fluorescent Protein (CFP) fused to a peroxisomal targeting signal (SKL). Its expression provides real-time confirmation that all of the steps required for gene expression have been completed (Janicki et al., 2004; Tsukamoto et al., 2000) (Figure 1). The concentration of CFPSKL in the peroxisomes both improves the signal-to-noise ratio during imaging and segregates it from the nucleus so that CFP can also be used to tag transcriptional regulators for simultaneous multi-color imaging.

Transcriptional Activators: Modular Tools for Probing Chromatin Organization

The transcriptional activator used in this system [tetracycline transactivator (tTA)] consists of a fusion between the bacterial DNA-binding protein, tetracycline repressor (TetR), and the HSV-1 VP16 transactivation domain (TAD)(Gossen and Bujard, 1992) (Figure 1 and Figure 2B). In the absence of tetracycline and its derivatives (e.g. doxycycline), tTA binds constitutively to the tetracycline response elements (TREs) in the transgene and initiates transcription from the minimal CMV promoter. When the activator is fused to an auto-fluorescent protein, such as Cherry, it permits the specific visualization of transcriptionally active chromatin (Figures 1). Tighter transcriptional control can be achieved by fusing tTA to the 4-hydroxytamoxifen (4-OHT)-inducible hormone-binding domain of the estrogen receptor (ER), which sequesters it in the cytoplasm prior to activation (Figure 1). In the absence of 4-OHT, ER associates with heat shock protein 90 (Hsp90) (Eilers et al., 1991). When 4-OHT is added to the media, ER sheds Hsp90 and enters the nucleus.

Due to its specific interaction with active chromatin, the Cherry-tTA-ER activator can be used to determine the timing of transcriptional events. Cherry-tTA-ER is first detected at the CMV-promoter-regulated transgene array in the U2OS cell line (2-6-3) ~7 min after the addition of 4-OHT to the media (Figure 2A). The simultaneous detection of the RNA, marked by YFP-MS2, with Cherry-tTA-ER indicates that, in U2OS cells, transcription is rapidly induced (Rafalska-Metcalf et al., 2010). Therefore, the activation time includes the time required for Cherry-tTAER to enter the nucleus and accumulate to detectable levels at the array. Simultaneous imaging of Cherry-tTA-ER with YFP-tagged regulators (e.g. Brd4, GCN5, and histone H3.3) can be done to determine the timing of their recruitment. Modeling these types of data can suggest novel mechanisms of gene regulation (Darzacq et al., 2007; Newhart et al., 2013b; Rafalska-Metcalf et al., 2010; Zhao et al., 2011).

The modular structure of the transcriptional activator lends itself to development as a tool for comparing the chromatin decondensation and transcriptional activation potentials of different cellular and viral TADs. The Cherry-TetR-(TAD)-ER construct was designed to construct new activators by directional cloning (Figure 2B) (Rafalska-Metcalf et al., 2010). Comparison of the strong VP16 and weak p53 TADs in the U2OS cell line (2-6-3) demonstrated that their differential effects on transcription and chromatin organization are inherent to their amino acid sequence, which likely regulates the levels and types of co-activators recruited (Rafalska-Metcalf et al., 2010).

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Exploring the Interface Between Transcription and Nuclear Organization

Numerous multi-subunit transcriptional regulators have been identified through biochemical and genetic analyses. The single-cell imaging strategy described in this review provides the opportunity to directly relate factor recruitment to changes in chromatin organization and RNA accumulation at molecularly defined transcription sites (Darzacq et al., 2007). Knockdown analyses can be combined with conventional techniques to validate the visual data. For example, strand-specific quantitative RT-PCR, high-throughput sequencing, and RNA FISH have been used to analyze RNA expression (Newhart *et al.*, 2013b). Chromatin immunoprecipitation has been used to monitor factor recruitment and histone post-translational modifications (PTMs) as well as to identify sequence specific enrichment (Newhart et al., 2013a; Newhart et al., 2012; Shanbhag et al., 2010; Tang et al., 2013; Zhao et al., 2011). Validated hypotheses about gene regulatory mechanisms can be tested at endogenous genes.

De Novo Transcription Sites: Giving Genes a "Fresh Start"

In addition to providing a previously unavailable view of transcriptional dynamics, the ability to create *de novo* transcription sites has several other significant advantages. Since the CMV promoter-regulated transgene is inducible and its expression is not required for viability, it can be propagated in cells in an inactive state until the synthetic activator, Cherry-tTA-ER, is expressed (Figure 1). In this way, transcriptional activation can be tracked from a naive inactive chromatin state. Using the MS2 tag to visualize the expressed RNA also makes it possible to define the window of linear transcriptional increase. Examining the recruitment of factors to chromatin as it transitions to the active state has the potential to illuminate regulatory mechanisms.

The modularity of single-cell imaging transgenes, such as the one described in Figure 1, make it possible to construct new versions in order to directly examine the effects wild-type and mutated genetic elements have on transcription and chromatin organization. By inserting different promoters, it is possible to study the effects of cell cycle, developmental, and environmental stimuli (Hocine et al., 2013; Larson et al., 2011; Maiuri et al., 2011; Yunger et al., 2010). Gene-specific RNA processing events, including mRNA splicing, polyadenylation, RNA degradation and mRNA export, can be studied by introducing different 3′ sequence elements (Ben-Ari et al., 2010; Brody et al., 2011; Katz et al., 2012; Martins et al., 2011). Using this approach to analyze viral genetic elements could provide insight into intrinsic immunity and viral latency.

Oh What a Difference Genetic Background Makes

Transgene arrays can be integrated into different cell lines to investigate the effects of genetic background on transcription. For example, the CMV-promoter regulated transgene (Figure 1) is refractory to activation in HeLa cells, which express both the histone H3.3 chaperone, Daxx, and the SNF2-type ATP-dependent chromatin-remodeling factor, ATRX (Newhart et al., 2012). Daxx and ATRX regulate the replication independent (RI) incorporation of H3.3 into telomeres and centromeres (Drane et al., 2010; Goldberg et al., 2010). In contrast to HeLa cells, transcription begins ~7 min after activation in the ATRX-null U2OS cell line (2-6-3) (Figure 2A), which demonstrates the important role RI H3.3

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chromatin assembly plays in regulating transcriptional repression and heterochromatin organization (Rafalska-Metcalf et al., 2010). Prior to being identified as RI H3.3 chromatin assembly factors, Daxx and ATRX were known to be PML-NB components, viral targets for disorganization and/or degradation, and regulators of gene silencing (Boutell and Everett, 2013; Lallemand-Breitenbach and de The, 2010). The differential expression patterns of the CMV-promoter transgene in HeLa compared to U2OS cells are consistent with previous reports on Daxx and ATRX function.

Multi-Copy Transgene Arrays: Regulating Repetition

An important feature of the single-cell imaging system is that the transgenes integrate into cellular genomes as multi-copy arrays. Not only does this provide the DNA volume needed to visualize changes in chromatin organization during transcriptional activation (Janicki et al., 2004; Rafalska-Metcalf et al., 2010), but it also makes it possible to discern patterns of regulatory factor organization at the site. For example, H3.3 is strongly recruited to the activated transgene array in the ATRX-null U2OS cell line (2-6-3), despite the fact that chromatin assembly is blocked (Newhart et al., 2012). Importantly, H3.3 accumulates in regions that are distinct from the chromatin, marked by DNA-binding proteins including the activator, Cherry-tTA-ER (Figure 3A, **panels a-d**). The intensity profile (Figure 3, **panel d),** which graphs pixel measurements along the line drawn through the array (note the yellow line in enlarged inset in the merged image, panel c), shows that the H3.3-YFP and Cherry-tTA-ER peaks do not overlap. This suggests that when chromatin assembly is blocked, H3.3-YFP accumulates with factors that function upstream of nucleosomal deposition.

Analyses of the H3.3 localization pattern revealed that it accumulates with sense and antisense RNA at the active transcription site, which suggests that it is recruited to its incorporation sites through an RNA-mediated mechanism (Newhart et al., 2013b). RI H3.3 chromatin assembly is a highly conserved but incompletely understood genome regulatory mechanism. Daxx and ATRX are mutated in several neurological cancers and gain-of-function mutations in H3.3 have been identified in pediatric glioblastoma (Jiao et al., 2011; Molenaar et al., 2012; Schwartzentruber et al., 2012; Wu et al., 2012). Therefore, the ability to visualize the dynamic interaction of H3.3 with a transcription site at which RI chromatin assembly is blocked could provide new insight into both genome regulation and disease pathogenesis.

A large body of literature indicates that multi-copy transgene arrays are silenced as a result of transcription through inverted repeats (Calero-Nieto et al., 2010; Davis and MacDonald, 1988; Dorer and Henikoff, 1994; Garrick et al., 1998; Henikoff, 1998). As the methods used to isolate these array-containing cell lines do not control for orientation, it is likely that they all contain inverted repeats. Although transcription is rapidly induced when Daxx and ATRX-regulated RI H3.3 chromatin assembly is disrupted (Figure 2A), the effects of transgene repetitiveness and inversion are not yet fully understood. In ATRX-null U2OS cells, the inactive CMV-promoter-regulated array is highly condensed, which suggests that additional mechanisms may function to compact the chromatin in order to preserve genomic integrity (Figure 1; **Cherry-lac repressor image**). Indeed the condensed structure of the inactive array is itself an impediment to transcription because the weak p53 TAD construct

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cannot induce decondensation to the same extent in the U2OS cell line as Cherry-tTA-ER, which contains the strong VP16 TAD (Rafalska-Metcalf et al., 2010). Therefore, this system provides an important opportunity to study the mechanisms which regulate repetitive DNA in mammalian cells.

Deciphering the Connection Between PML-NBs and Transcription

Overall, our analyses indicate that *trans*-acting factors (i.e. Daxx and ATRX) have a dominant effect on transcription compared to *cis*-acting elements (i.e. the genomic site of the array integration and the transgene repeats). For example, all of the CMV-promoter regulated transgene arrays independently integrated into ATRX-null U2OS cells (e.g. 2-6-3 and 60-1) (Newhart et al., 2012) (smj unpublished data) have been rapidly activated. In contrast, the array is refractory to activation in HeLa cells (cell line HI 1-1) unless ICP0, which depletes Daxx, ATRX and PML, is co-expressed (Newhart et al., 2012). PML-NBs are defined by the presence of PML protein (Lallemand-Breitenbach and de The, 2010). Therefore, the enrichment of PML at the transgene array (Figure 3B), suggests that it is a PML-NB and that PML-NBs are transcription sites. ChIP analyses indicate that Daxx, ATRX, PML and Sp100 are all specifically enriched at the CMV promoter (Newhart et al., 2013a; Newhart et al., 2012). This suggests that promoter sequences seed their assembly at the site. What is not known, however, is whether PML-NB proteins are recruited to specific promoters (e.g. viral promoters such as CMV) or all promoters in repetitive arrays. Overall, these results demonstrate the power of this imaging strategy for deciphering mechanisms of transcription, nuclear organization, and innate immunity.

Future Directions

The inherent features of this experimental system lend it to continual development as a discovery tool. Below is a discussion of the ways in which it can be expanded and refined.

Biochemical Purification—The protein/nucleic acid interaction units used for DNA and RNA visualization (Figure 1) can be adapted (e.g. immunological or streptavidin-based tagging systems) in order to purify the transgene chromatin and expressed RNA for comprehensive analysis of associated factors, PTMs, and RNA.

Targeted Integration—Genomic location is extremely important for its function. Additionally, it is desirable to visualize regulatory events at single-copy genes because the effects of the arrays on transcription are not fully understood. Therefore, site-specific homologous recombination can be used to create cell lines with single copies of these transgenes (Yunger et al., 2010; Yunger et al., 2013b). As detection sensitivity increases, it will also be possible to use high-resolution imaging methods to visualize regulatory factors dynamics at single-copy sites (Cisse et al., 2013; Gebhardt et al., 2013).

Using Transgene Engineering to Study Transcriptional Mechanisms—As mentioned above, the modularity of this transgene makes it possible to introduce different cellular and viral wild-type and mutated genetic elements into it in order to study their effects on transcription and chromatin organization. The development of reporter constructs with RNA pol I and RNA pol III regulated transcription units could also bring new insight

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into the dynamics of these transcriptional machineries. Boundary elements could also be included in order to understand their role in regulating chromatin and nuclear organization.

Summary

The limits to our understanding of cellular regulation are directly related to the limitations of our experimental techniques. Insights gained from using new technologies must always be rigorously evaluated in order to effectively integrate them into existing regulatory paradigms. The ability to visualize molecularly defined transcription sites in single living mammalian cells now provides unprecedented access to information about the timing and spatial organization of transcription. Only by directly examining the dynamic interactions of factors with specific sequence elements will it be possible to fully understand how cells "translate" the information encoded in DNA into heritable patterns of gene expression.

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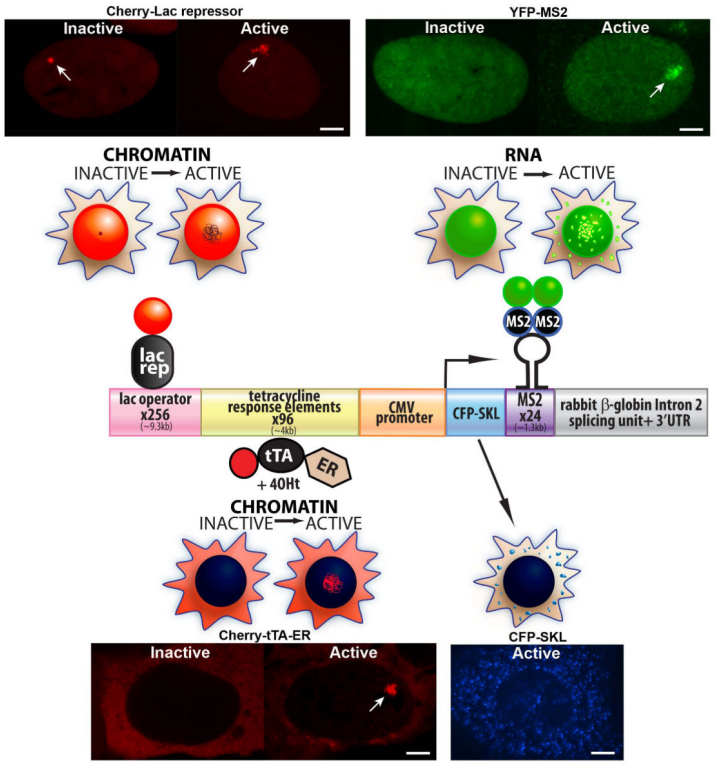

Figure 1.

Molecular and Cellular Characterization of the Single-Cell Imaging System The diagram of the single-cell imaging transgene shows the modular organization of its sequence components. The proteins, which permit the analysis of DNA and RNA in single living cells, are shown in association with the sequence elements to which they bind. The cell diagrams above each factor depict their localization patterns in inactive and activated cells. The cell images are of the U2OS cell line (2-6-3), which contains a multi-copy array of the CMV promoter regulated transgene. Cherry-lac repressor allows the transgene to be visualized in both the inactive (condensed) and active (deccondensed) states. Transcription is induced from the minimal CMV promoter by the activator, Cherry-tTA-ER, which is composed of the tetracycline transactivator (tTA) fused to Cherry and the estrogen receptor (ER) hormone-binding domain. Cherry-tTA-ER is retained in the cytoplasm until 4-hydroxytamoxifen (4-OHT) induces its entry into the nucleus where it accumulates at the transgene array and activates transcription. The transcription unit encodes Cyan Fluorescent Protein (CFP) fused to a peroxisomal targeting signal (SKL), MS2 bacteriophage translational operator repeats, and the intron 2 splicing unit of the rabbit β-globin gene. In inactive cells, YFP-MS2 is diffusely distributed in the nucleus. Upon activation, YFP-MS2 binds to the MS2 stem loop structure in the transcribe RNA both at the transcription site and throughout the nucleus. The enrichment of the protein product, CFP-SKL, in the peroxisomes provides real-time confirmation that all of the steps required for gene expression have been successfully completed. Scale bars =5 9m.

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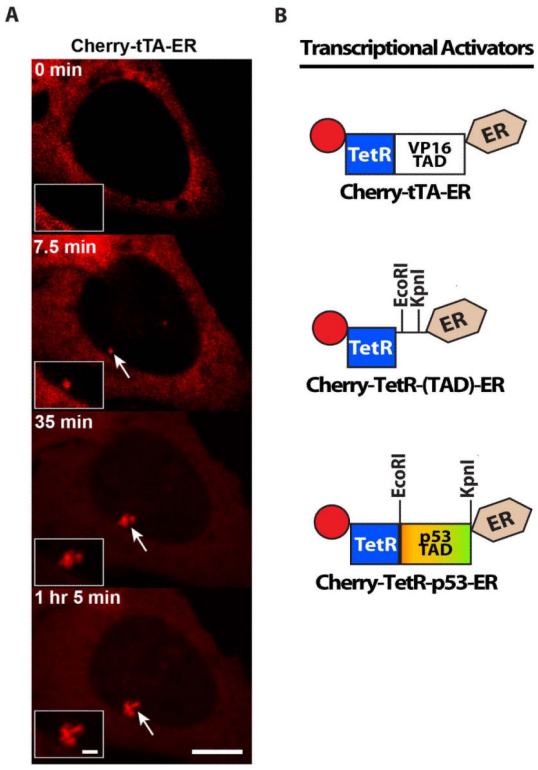

Figure 2.
Analysis of Active Chromatin Using Transcriptional Activators

A. Images from a time series of the recruitment of the activator, Cherry-tTA-ER, to the CMV-promoter regulated transgene array in the U2OS cell line (2-6-3). Arrows indicate the location of the array. Scale bar =5 ,m. Scale bar in enlarged inset =1 ,m.

B. (B) Schematic diagram of the synthetic transcriptional activator constructs, which can be used to analyze the effects of different transcriptional activation domains (TADs) on transcription and chromatin organization in cell lines with arrays that include the tetracycline response element repeats (Figure 1). Cherry-tTA-ER consists of the auto-fluorescent protein, Cherry, the tetracycline transcriptional activator (tTA), and the estrogen receptor hormone-binding domain (ER). tTA, itself, is a fusion between the tetracycline repressor (TetR) and the VP16 TAD. Cherry-TetR-(TAD)-ER was constructed for easy introduction of dirent TADs by directional cloning using EcoRI and KpnI. Cherry-TetR-p53 was constructed by introducing the p53 TAD into Cherry-TetR-(TAD)-ER.

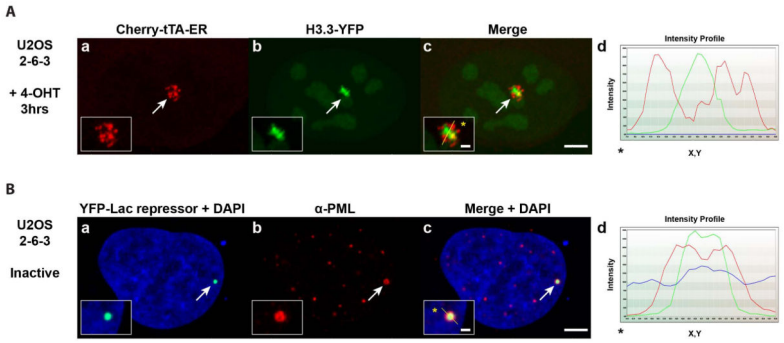

Figure 3.

Images of Regulatory Factor Organization at the Transgene Array.

(A) Histone H3.3-YFP (a) is strongly recruited to the activated transgene array in the ATRX-null U2OS cell line (2-6-3) in which replication independent histone H3.3 chromatin assembly is blocked. H3.3-YFP does not co-localize with the chromatin of the activated array marked by Cherry-tTA-ER (b). Arrows mark the location of the transgene array. Yellow lines in enlarged merge insets (c) show the path through which the signal intensities were measured in the intensity profiles (d). Asterisks mark the start of the measured line. Scale bar =5 0m. Scale bar in enlarged inset =1 0m.

(B) PML protein (b) is enriched at the inactive CMV-promoter regulated transgene array in the U2OS cell line (2-6-3), marked by YFP-lac repressor (a). DAPI DNA staining is shown in the merged images (a and c). (b)PML immunofluoresence staining was done with antibody PG-M3 (Santa Cruz Biotechnology).

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