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Dynamic regulation of transcriptional states by chromatin and transcription factors

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Abstract

The interaction of regulatory proteins with the complex nucleoprotein structures that are found in mammalian cells involves chromatin reorganization at multiple levels. Mechanisms that support these transitions are complex on many timescales, which range from milliseconds to minutes or hours. In this Review, we discuss emerging concepts regarding the function of regulatory elements in living cells. We also explore the involvement of these dynamic and stochastic processes in the evolution of fluctuating transcriptional activity states that are now commonly reported in eukaryotic systems.

The processes of development and differentiation in eukaryotic systems are regulated by constantly changing cohorts of site-specific DNA-binding proteins that direct cell-selective transcriptional programmes. A central paradigm in current biology argues that these factors interact with regulatory elements (that is, enhancers) to govern the activity states of target promoters in a cell-selective manner. In bacterial systems, site-specific recognition of DNA elements by regulatory proteins forms the central mechanism of promoter-specific regulation. However, in eukaryotes, particularly in mammals, simple site-specific binding is insufficient to govern the regulatory programmes given the large sizes and the complexity of their genomes. The organization of these genomes into complex nucleoprotein structures was originally thought to be only a packaging mechanism for DNA. It is now clear that chromatin provides markedly restricted access of transcription factors to regulatory sites in a highly cell-specific manner[1–4].

As cells replicate during differentiation, the range of elements that are available for binding by regulatory proteins constantly changes. This variable access to regulatory elements is now recognized to have a key role in normal cell development, as well as in altered expression profiles that are associated with many disease states[5–8]. A central question becomes how DNA-recognition proteins interact with the cellular enzymes that act on chromatin to either demarcate elements for action or silence these elements in a given cellular context. Although chromatin transitions can occur at many levels of biological organization, the aspects of chromatin structure that impinge on epigenetic regulation can be

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divided into three general areas. First, specific histone modifications have been widely studied, and there are clear subsets of histone marks that are associated with altered activity states for both promoters and enhancers. Second, as chromatin structures inhibit access to the underlying DNA sequence, selective localized access to regulatory elements (that is, 'open' chromatin) has emerged as a common feature of active regions[9,10].Third, long-range interactions between enhancers and targets occur on a wide scale; the chromosome conformation capture methodologies in various implementations open new windows for studying the role of nuclear architecture[11].

Although cell population-based approaches often show fairly static 'snap-shots' of chromatin architecture, the molecular processes that govern these states (such as nucleosome remodelling, transient protein–protein contacts and post-translational modifications) involve fast enzymatic reactions, which leads to fluctuations on multiple timescales[12,13]. The study of these real-time mechanisms is difficult and has thus received less attention. However, in the past decade, advances in the characterization of transcription factor action in living cells revealed an unexpected mobility of interactions of these factors with genomic sites[14]. Furthermore, single-cell studies of gene function have uncovered complex, nonlinear transcriptional programmes. Findings from these new approaches indicate that our previous understanding of transcriptional regulation grievously underestimates the complexity of this central biological process. There is an increasing awareness that complex dynamics on multiple timescales is central to the activation of appropriate transcriptional programmes. In particular, studies that reveal many rapid and cycling molecular processes emphasize the central role of time-dependent events, which we refer to as the 'fourth dimension' of gene regulation[15,16].

In this Review, we first discuss current concepts of the direct interaction between regulatory proteins and the chromatin fibre. We present alternative views concerning mechanisms by which these factors can overcome structural barriers that are intrinsic to nucleosome arrays and place a particular emphasis on the dynamic role of ATP-dependent remodelling proteins. We contrast the fairly static view of long-lived binding events with an emerging view of transcription factor action that is based on rapid template interactions, and we present models that integrate these concepts. We then consider how these mechanisms can be integrated to produce fluctuating levels of transcriptional activity.

Transcription factors and chromatin access

To successfully interact with genomic regulatory elements, transcription factors must induce the reorganization of local nucleosome structures. These disturbances of the ordered nucleosome array can be mapped by detecting DNA sequences that have increased nuclease accessibility, which are known as DNase hypersensitive sites[9,17–21]. These 'chromatin-penetrating' events are highly cell specific and determine, to a great extent, cell-selective transcriptional programmes[10,22,23]. The key issue then becomes how these local regions of chromatin access are identified and targeted.

Given the almost total reliance on chromatin immunoprecipitation followed by sequencing (ChIP–seq) and on similar population-based approaches, data sets are, by their nature,

averaged across highly heterogeneous cellular states. However, it is now clear that mechanisms in living cells show a dynamic dimension that is undetected in experimental approaches that inhibit these processes.

In vivo *dynamics of transcription factors.*

The characterization of real-time interactions of transcription factors with response elements led to the unexpected finding that residence times on genomic elements can be short[14]. Subsequent investigations showed that rapid exchange is a property of many transcription factors[24,25], although some site-specific DNA-binding proteins seem to have longer residence times[26–28]. A careful examination of the methodologies that are used to study real-time movement in living cells — including photobleaching (such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP)), fluorescence lifetime imaging (FLIM), fluorescence correlation spectroscopy (FCS) and single-molecule tracking (TABLE 1) — reveals several difficulties in the interpretation of these data sets[29,30]. However, it is now well-established that a large proportion of factors have high mobility on genomic sites. The mechanisms that underlie these rapid binding and unbinding events are undoubtedly complex, and they constitute a major challenge for the research field.

These observations of rapid exchange are in sharp contrast with the classic view of long-term residency for transcription factors. For example, if regions of nuclease hypersensitivity are sequenced to great depths, classic footprints that correspond to potential transcription factor-binding sites will appear[22,31]; these results are usually interpreted as DNA protection due to binding of a factor. However, a careful examination of binding elements for a given factor throughout the genome reveals patterns that are, in some cases, inconsistent with simple static binding. As an example[22], elements that show equal levels of transcription factor AP-1 binding by a ChIP–seq analysis sometimes produce marked footprints at some sites but reveal no footprint at all at other sites. The simplest basis for the absence of footprints is tethering of one protein to a second, DNA bound, factor[32,33]. However, for proteins that directly bind to DNA, the molecular processes that produce a certain level of factor which is detected at a given site are likely to be more complex than simple static DNA binding. In some cases, dynamic binding and unbinding may be responsible for the failure to observe footprints at sites with high levels of occupancy indicated by ChIP–seq analyses. Indeed, it is likely that transcription factors show a broad range of actual residency times on the template.

In vivo *dynamics of nucleosome remodelling.*

The local disruption of nucleosome structures that accompanies transcription factor binding is, in many cases, known to be associated with the recruitment of specific ATP-dependent remodelling systems[34]. Several molecular mechanisms are involved in nucleosome reorganization by these systems[35–39], including nucleosome sliding and displacement, partial histone disassembly and substitution by histone variants (FIG.1a). Similarly to factor occupancy, it is commonly assumed that these reorganization events result in the conversion of one nucleosome state to an alternative state with long lifetimes (FIG. 1a). Several mechanisms have been proposed to account for the modulation of factor access that accompanies these chromatin reorganization events.

Cooperative nucleosome attack.

The least complex model for initiating access to nucleosome-protected sequences suggests that two or more factors that act in tandem could dislodge the histone octamer or its sub units (FIG. 1b). In this model, the combined binding energy of two or more factors is argued to be sufficient to overcome the many protein–DNA contacts that stabilize the nucleosome structure. Although versions of this general model have been discussed for many years[40–42], there is little direct evidence to support this mechanism.

Pioneer proteins.

An alternative mechanism for factor access invokes a special class of factors that are designated pioneer proteins (FIG. 1c). These factors are postulated to have unique properties that allow them to interact with closed nucleosome arrays, thereby creating access for factors with binding sites that are co-incident with the opened site. Frequently discussed members of this class include those of the forkhead box protein A (FOXA) and FOXO families[43–45]. The FOXA family has been widely implicated as a pioneer factor for the oestrogen receptor[46], and the involvement of this protein in human cancer has been suggested[47].

The molecular mechanism by which these factors modulate local nucleosome structure is not yet clear. One study showed, using *in vitro* assembled nucleosome arrays, that FOXA1 could create a nuclease hypersensitive region in the array[48]. In this work, similarities were noted between the structures of FOXA1 and histone H1, which suggests that the winged helix domains in FOXA proteins could disrupt DNA–histone contacts, thereby creating accessible transcription factor-binding sites (FIG. 1c). Neither ATP-dependent remodelling factors nor chaperone proteins have been invoked in these mechanisms.

As discussed above for footprints, these models envisage long-lived nucleosome states that provide altered factor access. However, given our increased awareness of rapid exchange that is associated with factor binding in living cells, the question arises as to the potential involvement of chromatin remodelling in these dynamic processes (FIG. 1d).

Mobility during remodelling.

detailed statistical analysis for remodelling of the N1, N2 and N3 nucleosomes at the *PHO5* locus in yeast[49,50] concluded that induction of these remodelling events by phosphate depletion produced a shift in the equilibrium of nucleosome assembly and disassembly, rather than a conversion of a static occupancy state to a second static depleted state. This analysis emphasizes a dynamic process rather than a transition between static states.

Two *in vitro* studies further illustrate the complexity of this issue. High-efficiency UV laser crosslinking was used to characterize template occupancy by the glucocorticoid receptor during remodelling, which is catalysed by the SWI/SNF complex[51] (FIG. 2a). Receptor binding was found to be periodic during the remodelling process. Each burst of receptor recruitment was rapidly followed by eviction of the receptor from the template. Furthermore, the periodic binding behaviour was completely reliant on ATP-dependent remodelling. This finding suggests a two-step process. Although receptor–SWI/SNF interactions are required

to bring the remodeller to the correct position on the template, some component of the remodelling reaction then leads to displacement of the receptor.

A second example directly focuses on the displacement phenomenon. A synthetic nucleosome remodelling system was established, whereby a yeast chromodomain-containing protein 1 **(**Chd1) remodeller domain was fused to the DNA-binding domain of arabinose operon regulatory protein (AraC), which is a *Escherichia coli* transcription factor[52] (FIG. 2b). This fusion protein, similarly to many other remodellers, catalyses movement of the nucleosome to the centre of the template. When a template without the AraC-binding site is used, the fusion remodeller is free to diffuse and is never anchored to the template. However, when it is recruited to the template by the AraC domain, a trimeric complex is observed. When nucleosome sliding is then induced, the nucleosome actively displaces the fusion protein from the template. Although this experiment uses a highly synthetic system, it clearly shows the consequences of histone octamer movement through factor-binding regions (that is, 'clash of the Titans'). Other examples of this phenomenon have been described for disparate remodelling systems[53]. The phenomenon provides a mechanistic basis for factor mobility that has been observed *in vivo*.

The assisted loading model.

If factors are bound to their response elements in living cells with lifetimes that are similar to those seen on pure DNA templates *in vitro*, then competitive displacement should be observed for proteins that bind to the identical sequence. This concept was recently subjected to direct testing[54]. When two factors that bind to the same site but that are activated by different ligands were expressed in living cells (FIG. 1d), they failed to compete for the binding site. Furthermore, activation of one factor actually increased binding for a second factor when it was tested, throughout the genome, at sites that correspond to endogenous response elements for the first factor. Importantly, this phenomenon (which is designated assisted loading) only occurred when the first factor (that is, the initiating factor) was responsible for chromatin opening at the endogenous sites tested; that is, *de novo* opening of chromatin by the initiating protein is necessary to observe assisted loading. It is proposed that the recruitment of remodelling systems by the initiating factor leads to a transient opening of chromatin at the response element, which provides a 'window' in time, during which secondary proteins gain access to their binding sites (FIG. 1d). However, as discussed above, the residence times for individual factors are brief, and competition is therefore not seen under these circumstances. In contrast to the pioneer-protein framework, this model suggests that many transcription factors can be 'initiators' of enhancer activation. The key determinant is the status of the local chromatin domain, rather than a special property of the initiating factor.

Resolving co-binding transcription factors

The dynamic mechanisms discussed above are in contrast to the static models that are frequently invoked for enhancer function. Two difficult issues are resolved. First, the dynamics of factor mobility on chromosomal sites *in vivo* is rigorously accounted for by the assisted loading model. Second, the density of factor-binding sites in regulatory regions has

been a long-standing problem (FIG. 3a). Indeed, the application of ChIP at the genome scale reveals that factors are often found in dense, localized clusters, which are often referred to as transcription factor hot spots[20,31,55–59]. There are three general solutions to this problem. Conceivably, factors could be organized in large multimeric complexes at hot spots, where some of these factors are bound to DNA and some are localized only by protein–protein interactions (FIG. 3a). Indeed, multifactor complexes have been well-described[60]. This cannot be a general solution, as there are well-documented examples[61] in which factors that co-bind *in vivo* actually compete for binding when tested on pure DNA *in vitro*. Alternatively, there could be cell subpopulations in which only a subset of the factors are bound (FIG. 3a). There are two versions of this model. On the one hand, one could propose that successive factor sets are sequentially recruited to a given site; when such recruitment events are averaged across the population, these proteins would seem to be co-resident. On the other hand, there could be alternative paths of factor recruitment at a given site in different cells; this version seems unattractive, as it is difficult to theorize why the same factors would behave differently in different cells. A third model would envision multiple transient states, in which factors move dynamically on and off the template (FIG. 3a). Some of these states could have relatively long lifetimes compared with others.

A theoretical view of this dynamic model is shown in FIG. 3b,c. Cells in a given population are heterogeneous with respect to the precise molecular status of promoters and enhancers. Complex multifactorial states are averaged by any population-based biochemical assay. ChIP–seq signals, in particular, must originate from a range of molecular states. Furthermore, it is likely that chromatin-remodelling complexes participate extensively in the dynamic events that are associated with transcription factor binding (FIG. 3b). Unfortunately, ChIP-grade antisera that are specific for these proteins are not widely available, and fairly little is known about the genome-wide distribution of these complexes. However, a recent study reported that multiple remodelling proteins can function at a given enhancer[62], which is consistent with dynamic intermediate states.

A central question concerns the potential cyclical nature of these processes (FIG. 3b). Nucleosome reorganization and factor binding may proceed through a linear series of steps, with each step serving as a deterministic substrate for subsequent events. Alternatively, these processes may be cyclical, in which factors initiate continuous nucleosome assembly and disassembly.

A further complexity is suggested by the dynamic model of enhancer function. A standard assumption is that static complexes (FIG. 3a) produce long-lived enhancer states that interact over long distances with target promoters. We speculate that the intermediate states that are produced during nucleosome cycling give rise to separate and distinct interactions with their targets (FIG. 3b); that is, a given enhancer may induce different effects as it evolves through multiple states.

Regulating dynamic gene expression

Temporal patterns of gene expression.

The dynamic action of regulatory proteins described above is integrated at the cellular level into distinct temporal expression patterns. These cellular responses can be grouped into related types (FIG. 4a). If the response shows a variable pattern that does not repeat consistently over time, then this may be due to a probabilistic or stochastic behaviour in the cell or the surrounding environment[63,64]. The presence of reproducible oscillations suggests that there is some ordered mechanism in the biological system that produces this deterministic behaviour[65]. There are also examples of reproducible temporal gene expression that generates peaks of activity; that is, such gene expression is either monophasic or biphasic[66,67]. In higher organisms these complex patterns can be generated at the levels of the intact tissue, the individual eukaryotic cell and the regulatory chromatin[15] (FIG. 4b). Time-dependent regulation that originates inside the individual cell is described as cell intrinsic[15,68,69]. Complex network feedback among specific intracellular signal transduction pathways produces these distinct temporal trends in expression[70,71]. These mechanisms are in contrast with cell-extrinsic regulation that arises from dynamic changes in the environment that surrounds the cells. It is important to note that both intrinsic and extrinsic processes should be explicitly defined in a context-specific manner. For example, time-dependent patterns can also originate at the level of a single promoter or enhancer (that is, at the level of an individual allele). In this context, the intracellular signal transduction networks are extrinsic to the individual alleles.

The mechanisms discussed above are likely to be modulated to create temporal expression patterns in eukaryotic cells through both cell-intrinsic and cell-extrinsic mechanisms. The experimental data that are currently available force the reconciliation of two general properties, which at first may seem to be at odds with each other. In the averaged cell population, transcriptional responses are often reproducible and seem to be deterministic. However, single-cell and single-molecule approaches reveal the stochastic and/or probabilistic nature of the underlying mechanisms. The combination of regulatory chromatin and the transcriptional apparatus integrates multiple signals over time and unites both deterministic and stochastic behaviour. Below, we discuss several examples that show the interplay between deterministic extrinsic signals and intrinsic probabilistic responses at the level of chromatin.

Transcriptional oscillations due to pulsatile, cell-extrinsic signals.

Cells must quickly respond to cues from their external environment. These signals are transmitted from one cell to another in higher organisms by the release of endocrine or paracrine hormones. During organismal development, time-dependent hormone concentration patterns evolve over extended periods. However, hormone secretion is also commonly pulsatile in nature, and this produces additional time-dependent oscillations of ligand concentration that the specific receptor proteins in target cells are subjected to (reviewed in REF. 72). Ultradian hormone pulses occur multiple times per day and range over durations that last from sub-seconds to several hours. For both nuclear receptor and membrane-bound receptor pathways, many of the activated signal transduction mechanisms

ultimately control the expression of specific target genes. Therefore, it is hypothesized that the involved DNA-binding transcription factors (DBTFs), target chromatin and core transcriptional machinery quickly adapt to rapidly fluctuating hormone signals.

Live-cell imaging of interactions between GFP– glucocorticoid receptors and target chromatin clearly showed the dynamic chromatin response to transient and pulsatile extracellular signalling[73]. Within ten minutes of treatment with corticosterone (which is a natural receptor ligand), the steroid receptor interacts with response elements in the mouse mammary tumour virus (MMTV) promoter and stimulates recruitment of RNA polymerase. As discussed above, activation of this promoter requires glucocorticoid receptor-dependent remodelling of the local chromatin. Within ten minutes of ligand removal, both steady-state glucocorticoid receptor–chromatin interactions and polymerase recruitment are reduced[74]. Importantly, this dynamic chromatin response to corticosterone pulsing is also observed by ChIP methods at endogenous glucocorticoid receptor-target chromatin sites in both cell line models and intact animals[73]. Subsequent studies reinforce the concept that the timing of pulsatile inputs controls the transcriptional output in diverse systems[75–77]. By monitoring the expression from individual cells and by artificially adjusting the pulse-frequency of an extracellular signal in real time, both the average level of reporter gene expression at various time points and the level of stochastic variance between individual cells could be controlled[78]. Thus, the regulatory chromatin and transcriptional apparatus clearly respond to varying pulsatile input signals and, as a result, produce distinct expression patterns.

Temporal transcription patterns due to cell-intrinsic feedback loops.

Several essential transcriptional regulatory systems contain multiple intracellular signalling loops that add temporal complexity to the response from individual cells, and this is probably a general feature of transcriptional control[71]. Through this cell-intrinsic mechanism, a single transient or sustained extracellular input can initiate multiple oscillations and/or time-dependent transcription from specific target genes. Nuclear factor-κB (NF-κB) and p53 are two robust examples of DBTFs that are involved in this type of modulation. To mediate the cellular response to stress or inflammatory signals, NF-κB translocates from an inactive form in the cytoplasm to the nucleus, where it serves as a DBTF (reviewed in REF. 79). In response to the initiating extracellular event, IκB proteins are phosphorylated and are rapidly degraded. In the absence of interacting IκB, the remaining NF-κB heterodimer (which consists of p65–p50 family members, for example, RELA–NF-κB1) concentrates in the nucleus and regulates transcription of many target genes. The negative-feedback loop is closed when the NF-κB heterodimer stimulates expression of the gene that encodes IκB, which in turn causes increased concentrations of IκB to sequester the NF-κB heterodimer in the cytoplasm. The period of NF-κB oscillations can vary over the timescale of minutes to hours and dynamically regulates downstream gene expression[80,81]. Owing to asynchronous behaviour between individual cells, these patterns are difficult to measure through biochemical methods that average the behaviour of the whole cell population.

The response to DNA damage or to other cellular stress through the p53 pathway can generate similar time-dependent changes in gene expression[82]. The active p53 in the nucleus

is dynamically regulated though functional interactions with negative-feedback loops that involve multiple genes and with their associated signal transduction pathways. Depending on the nature of the extracellular signal, oscillations of both p53 and NF-κB activities can vary in amplitude, frequency and synchronization among individual cells[83,84]. These and other similar feedback-driven signal transduction and DBTF systems suggest that the target regulatory chromatin structure and the core transcriptional machinery can dynamically adapt to accept these inputs[84].

Interestingly, some regulators, which are known components of intracellular feedback loops that operate upstream of individual alleles, behave in a stochastic manner (FIG. 4c). However, computational modelling predicts that the time delays, which are involved in various steps of a negative-feedback loop, can produce oscillations in individual cells even when the underlying events are stochastic in nature[85]. This has been shown in several experimental studies. For example, stochastic calcium spikes cause nuclear translocation of Crz1, which is a DNA-binding transcription factor, and activation of its downstream target genes[86]. These results show that the frequency but not the duration of the calcium spikes controls the overall pattern of translocation and the resulting transcriptional activity. Another DBTF — zinc-finger protein Msn2 — also stochastically translocates into the nucleus[86,87], which suggests that stochastic translocation is a common feature of signalling cellular pathways that control transcription. Different types of cellular stress drive the translocation with specific characteristics over time. Downstream genes respond to these different translocation patterns with specific transcription and expression patterns.

Stochastic chromatin interactions

In either bacterial and archaeal or eukaryotic single cells, transcription commonly occurs in a discontinuous manner over time[69,88–94]. These diverse studies suggest that some of the transcription-regulating events occur asynchronously and produce a large degree of heterogeneity in the cellular output. The heterogeneous activity can be derived from small numbers of interactions between regulatory DNA and DBTFs[64,68]. This allele-intrinsic mechanism is a key aspect of stochastic transcription and generates some of the biological noise in the gene expression process. Classical deterministic kinetic models do not explain these erratic properties, but several quantitative stochastic and/or probabilistic models provide mechanistic insights for the observations (reviewed in REFS 95,96).

Evidence for stochastic transcription.

The MMTV long terminal repeat (LTR) is one of the key systems that facilitated the discovery of stochastic transcription in individual mammalian cells[97]. Even among clonal cells, this promoter produces highly heterogeneous expression that is not due to differences in cell cycle stage. When the glucocorticoid ligand signal is maintained at constant levels, the MMTV LTR creates a glucocorticoid receptor-inducible biphasic transcriptional pattern over time at the averaged cell population level[98,99]. This single-cycle transcriptional pattern (that is, either monophasic or biphasic glucocorticoid receptor-dependent activation or repression) is also observed from many endogenous glucocorticoid receptor-responsive genes[66]. By contrast, the activity of several oestrogen receptor-dependent genes oscillates in

a multicycle manner[100,101]. The transcriptional activity temporally correlates with increases in steady-state binding of both oestrogen receptor and its recruited co-activators to the regulatory chromatin. However, recent studies have not confirmed the multi-cycle nature of the oestrogen receptor response[102]. Importantly, studies of a copper-induced yeast gene show that both multiple cycles of transcriptional oscillation and its correlated steady-state factor recruitment (in which each oscillation lasts for several minutes) coincide with rapid flux (on the timescale of seconds) of DBTFs and cofactors at the regulatory chromatin[103]. The stochastic characteristics of these promoters and enhancers remain to be quantified. However, this range of time-dependent transcriptional patterns raises the question of how stochastic transcriptional mechanisms are regulated to produce the reproducible behaviours that are observed for the averaged cell population measurements.

Resolving stochastic transcription.

The ‘two-state’ chromatin model is useful as a conceptual and quantitative framework for characterizing stochastic transcription[69,92]. In this paradigm, the enhancer and/or the promoter of a particular gene undergo a transition between an ‘on’ state and an ‘off’ state with some defined probability per time interval. Transcription initiation and the production of mRNA are determined by a second probability variable, and this probability is increased when the enhancer and/or the promoter is in the on state. This simple model accounts for both transcriptional bursting at individual alleles within a single cell and gene expression hetero geneity between isogenic cells. The physical differences in the on and off states of target genes probably involve changes in conformation of the regulatory chromatin[50,69,104,105]. Although there may be many different local chromatin modifications, if one of them is rate-limiting then the system may respond as if there is a single chromatin state transition. Importantly, both regulatory sequence and chromatin modification status alter the probabilistic transcriptional responses from specific mammalian genes[105]. Interestingly, this model is highly compatible with the ‘hit-and-run model’ of transcriptional control (FIG. 4b,c).

Glucocorticoid receptor-inducible systems have produced substantial insights into how the stochastic patterns of gene expression are resolved into population-level patterns of gene expression. The average behaviour of individual MMTV LTR-containing cells over time seems to be consistent between experiments using microscopic or biochemical methods[67,99]. Steady-state interactions of both glucocorticoid receptors and recruited cofactors with regulatory chromatin are highly heterogeneous between individual cells. Moreover, steady-state glucocorticoid receptor–MMTV LTR interactions in individual cells only partially correlate with transcriptional activity, and steady-state binding of the glucocorticoid receptor is only partially correlated with recruitment of individual cofactors. These observations suggest that there are many different chromatin states and that transitions between these states are probabilistic. The frequency of unique DBTF–cofactor combinations is altered over time, which drives specific transcriptional outputs[106]. As the MMTV array system that is used in these studies contains many copies of the promoter–reporter unit, the array should buffer the apparent single-cell heterogeneity. However, this is not observed. Thus, the stochastic activating events may physically spread over a genomic region that encompasses several promoter–reporter units. These activating events may include large-scale chromatin

organization, looping, transcription ‘factories’ and/or a linear spread of nucleosome modifications. Alternatively, the source of the heterogeneity observed in the MMTV LTR system could be derived from the cell-intrinsic variability of an upstream signalling pathway.

Oestrogen receptor-inducible experimental systems also increase our understanding of the connections between stochastic events and patterned temporal gene expression. Similarly to the oscillating transcription pattern that has been reported for some other oestrogen receptor-regulated genes[100,101], the promoter and enhancer activity of the prolactin (*PRL*) gene also varies over time[107,108]. During basal *PRL* expression, oscillating peaks of transcription are asynchronous between individual clonal cells. When two different *PRL* promoter-driven reporter genes are integrated in the same cell at independent genomic locations, live-cell imaging under basal conditions reveals asynchronous oscillating expression of the two reporters[65]. Therefore, the generator of the pulse pattern acts at the level of the individual allelic *PRL*-regulatory chromatin regions. Treatment with either pharmacological agents that simulate physiological activation or an inhibitor of histone deacetylase activity temporarily synchronizes the *PRL* promoters and increases expression of the *PRL* reporter gene[65]. Live-cell imaging of engineered *PRL* regulatory chromatin also indicated an oscillating expression pattern[109], which was paired with hyperdynamic interactions between the oestrogen receptor and its regulatory elements, and more gradual changes in the steady-state association levels[25]. Thus, the events that control oestrogen receptor-dependent transcription operate at the mechanistic levels of the hyperdynamics and the stochasticity of interactions between the DBTF and its target chromatin, and of the oscillation of chromatin transitions.

The oscillating nature of the *PRL* transcriptional response is reinforced by a refractory period, which depends on dynamic chromatin remodelling events[65]. The enhancer and the promoter cannot be reactivated during the refractory period, which acts as a negative-feedback loop at the level of the individual allele. Again, a negative-feedback loop can generate reproducible temporal patterns from stochastic regulatory events[85]. Importantly, many eukaryotic genes show a refractory period, and different durations of latency drive promoter-specific activity patterns[105]. This refractory behaviour is lacking in bacteria, archaea and lower eukaryotes, which indicates that some aspects of higher eukaryotic expression patterns may be derived from more complex, long-range chromatin interactions and/ or chromatin remodelling events[12,110]. However, lower eukaryotes can also show reproducible transcriptional patterns that are predictable at some level. Therefore, the more general hypothesis is that a combination of multiple feedback loops, which may operate at several levels of intracellular regulation (FIG. 4b), arranges stochastic chromatin transitions into reproducible temporal transcriptional patterns.

Moving towards testable quantitative models

Our current perspectives on transcription regulatory mechanisms mostly derive from cell population-based biochemical procedures, transient transfection approaches using synthetic gene reporters, purification and characterization of factor complexes, and *in vitro* reconstitution with pure DNA templates. Developments in several emerging areas will contribute to a more rigorous understanding of the dynamic mechanisms that are involved in regulating gene expression. Major advances in fluorescence microscopy now offer access

both to single-molecule tracking approaches[111] and to the detailed time resolution of protein movement in individual cells[112–114]. We can expect continued evolution in these methods, which would provide crucial detail concerning molecular dynamics in living cells.

Although efforts to reconstruct transcriptional systems from chromatin are in their infancy, many of the central issues raised here can only be addressed with robust *in vitro* biochemical reconstitution. In particular, linking the action of specific transcription factors to the targeting of remodelling complexes lies at the core of enhancer and promoter function but has barely been addressed. There are more than 50 members of this important family, but global distributions of these complexes have not been addressed in any depth. Finally, a few studies have suggested that nucleosome remodelling at many sites involves an equilibrium between assembly and disassembly. If this concept is substantiated, then it would represent a major advance in understanding the dynamics of the action of regulatory proteins.

Conclusions

Transcriptional regulation has been treated as a deterministic process to a large extent, in which protein– protein and protein–DNA recognition drive processes inevitably through a series of highly ordered steps. With advances in technology and experimental approaches, we now understand that mechanisms have strong probabilistic components and that there are large variations in completion of individual steps at the single-cell or single-allele level. Furthermore, our inability to monitor events at high time resolution in living cells has masked a level of dynamic complexity in transcriptional mechanisms that is now beginning to emerge. As we decipher the details of real-time interactions between the hundreds of proteins that are involved at the chromatin– transcription factor interface, we will move to a more accurate understanding of the processes that are so central to gene regulation.

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DNase hypersensitive sites

Local regions of nucleosome reorganization that are detected by their increased accessibility to nucleases.

Fluorescence recovery after photobleaching

(FRAP). An optical technique to measure interaction lifetimesof molecular species. It involves labelling a specific cell component with a fluorescent molecule, followed by photobleaching a sharply defined region of the cell. Imaging is used to observe the subsequent rates and patterns of fluorescence recovery.

Fluorescence lifetime imaging

(FLIM). An alternative fluorescence method to measure lifetimes of molecular species.

Fluorescence correlation spectroscopy

(FCS). A fluorescence method to determine the average lifetime of the interaction between two molecular species when they are present within a given volume.

Single-molecule tracking

A method in which the path of a protein that is labelled with a bright chromophore is followed through the cell in real time.

Tethering

The localization of a protein to a specific site on DNA not by direct DNA binding but by interactions with another protein factor that is bound to the DNA; it is usually detected by chromatin immunoprecipitation.

ATP-dependent remodelling systems

Large multisubunit molecular machines that use ATP energy to reorganize nucleosome structures, often by sliding the nucleosome to a new position on the DNA.

Pioneer proteins

Factors that are proposed to have properties which allow them to penetrate local nucleosome structures and thus to pioneer the recruitment of secondary factors.

DMS footprinting

A method that detects regions of DNA resistance to chemical attack by dimethyl sulphoxide (DMS) owing to the presence of an interacting DNA-binding protein.

UV laser crosslinking

A method that uses dense pulses of high-energy ultraviolet (UV) photons to crosslink DNA bound proteins to the template rapidly and with high efficiency.

Assisted loading

A mechanism that proposes the transient recruitment of a transcription factor to the template by dynamic nucleosome remodelling.

Transcriptional bursting

The phenomenon whereby transcripts are sometimes released from activated promoters as rapid short pulses rather than in a continuous mode.

Hit-and-run model

The hypothesis that many transcription factors reside on specific binding sites for brief periods, which is followed by many of these factors returning to template cycles.

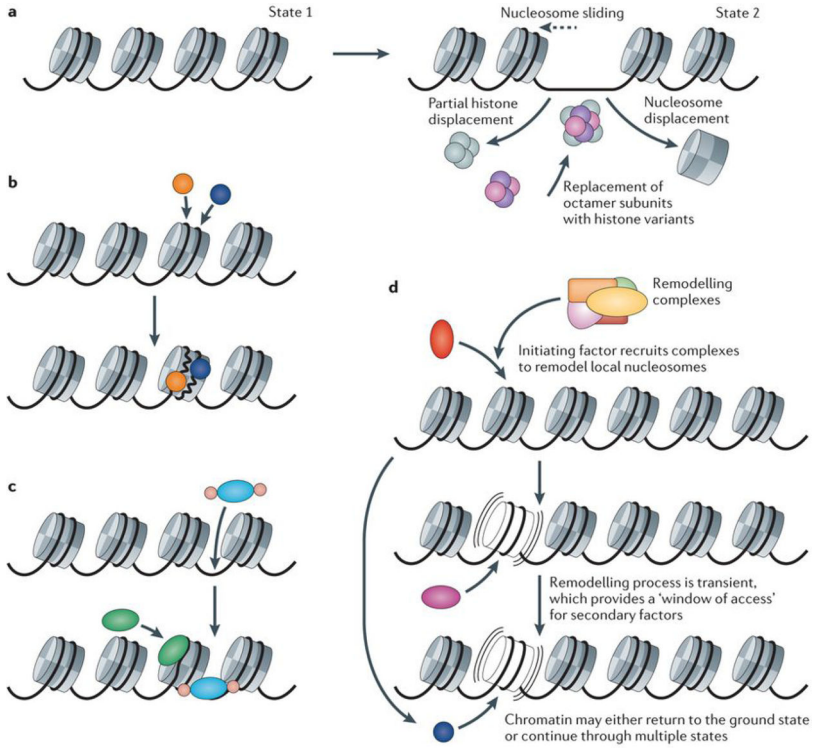

Figure 1. Models for chromatin penetration.

a | Most current models that invoke either ATP-dependent remodelling or histone chaperone action envisage a factor-dependent conversion of one static chromatin state to an alternative state. Possible mechanisms for increased local access include nucleosome sliding (shown by the dashed arrow), nucleosome displacement, partial histone displacement and replacement of octamer subunits with histone variants (shown in pink and purple)[35–39]. **b** | One of the classic mechanisms for factor penetration of closed chromatin structures is cooperative nucleosome attack. Although one protein may not be able to dislodge or reorganize local nucleosome structures, it has been suggested that two factors (shown in orange and blue) that act in tandem cooperatively generate sufficient free energy of binding to overcome the many histone–DNA contacts in the nucleosome[40], which leads to a new state with static factor binding. **c** | In another mechanism, pioneer proteins are argued to have special properties that allow their interaction with closed chromatin[43]. For the forkhead box protein A (FOXA) and FOXO families of proteins (shown in blue and pink, respectively), their structural similarity with histone H1 may lead to 'wedging' of DNA–histone contacts, which allows secondary access of other transcription factors (shown in green). Neither of these concepts invoke a necessary role for ATP-dependent remodelling complexes in the generation of altered nucleosome states. **d** | An alternative view suggests that the averaged states that are observed in cell populations result from a highly dynamic interconversion between multiple local chromatin states. A recent example of this concept is the assisted loading model for nuclear receptor action[54], in which receptors recruit multiple remodelling complexes to induce local chromatin conformations that have short lifetimes. These modulated states provide transient 'windows of access', during which secondary factors (shown in red, pink and blue) gain access to their binding sites. This process may either

continue in a linear mode, which generates a series of altered chromatin states, or involve continuous cycling.

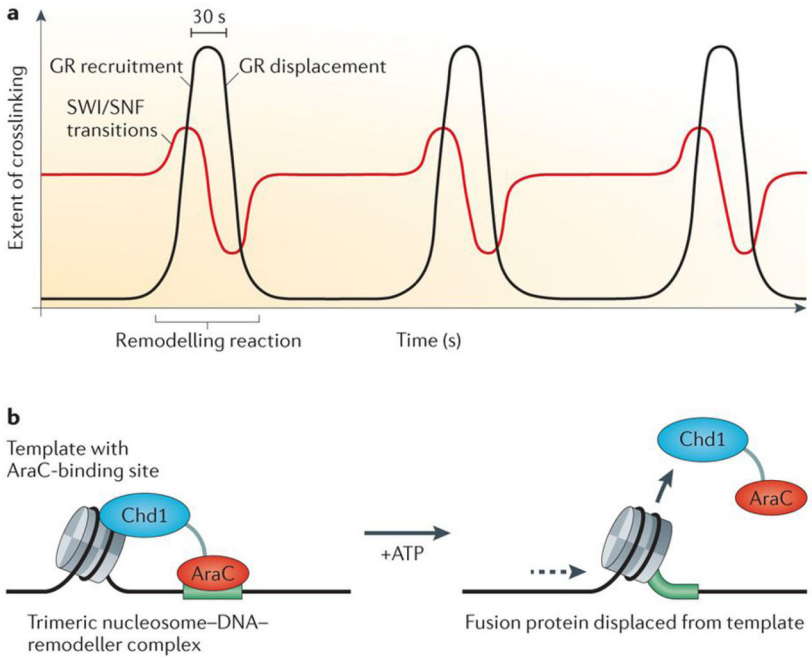

Figure 2. Transcription factor mobility.

a | The extensive reorganization of nucleosome structures during ATP-dependent remodelling can induce unexpected consequences for transcription factor binding. Glucocorticoid receptor (GR) binding during receptor-induced nucleosome B remodelling at the mouse mammary tumour virus promoter by the SWI/SNF complex[51] was studied *in vitro*, following real-time factor–template interactions with high-speed UV laser crosslinking. Pulses of GR recruitment and displacement (shown in black) were found to require ATP-dependent remodelling and to correlate with transient changes in BRG1–template crosslinking (shown in red). It was concluded that the receptor was actively ejected from the template during remodelling. **b** | One study[52] characterized transcription factor binding during nucleosome sliding that is induced by a fusion protein between the yeast chromodomain-containing protein 1 (Chd1) and the DNA-binding domain of the *Escherichia coli* arabinose operon regulatory protein (AraC). This synthetic system allows tethering of the Chd1 remodelling protein domain to a specific binding site (shown in green), which is mediated by the AraC DNA-binding domain. Sliding of a positioned nucleosome to the centre of the DNA template (shown by the dashed arrow) results in an eviction of the fusion protein from the template by nucleosome invasion of the binding site. This phenomenon shows how factor mobility can result from the large-scale macromolecular reorganization that occurs during chromatin remodelling.

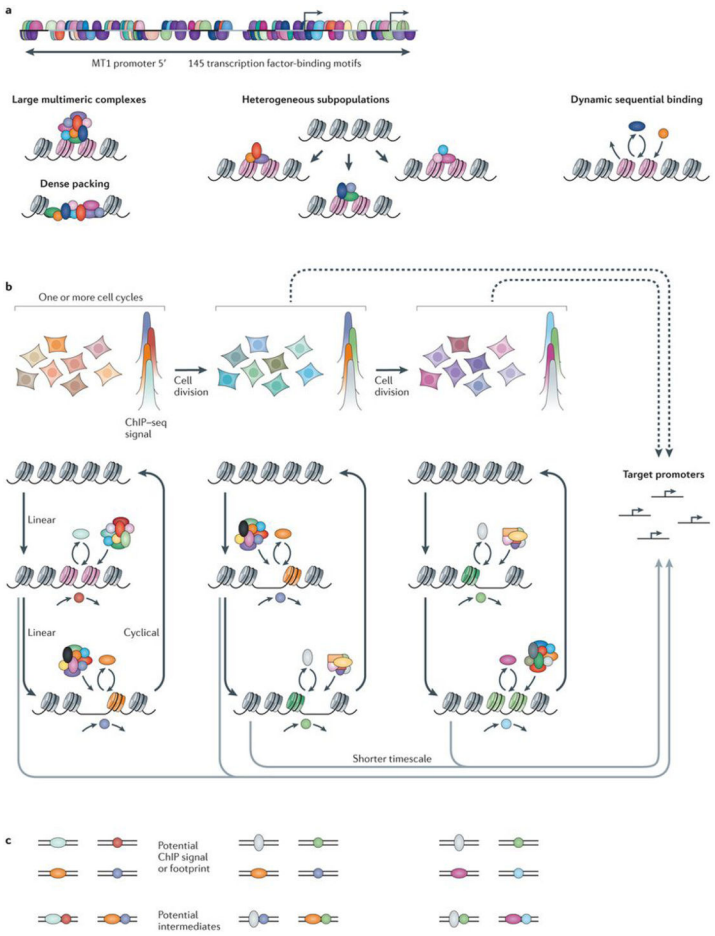

Figure 3. Dynamic enhancer function in mammalian cells.

a | Mammalian promoter and enhancer elements are densely packed with transcription factor-binding motifs. An analysis using the Genomatix software suite showed that the 756−bp region upstream of the metallothionein 1 (MT1) promoter contains 145 motifs, many of which are overlapping. In addition, a chromatin immunoprecipitation followed by sequencing (ChIP–seq) analysis reveals numerous localized elements that bind to as many as 40–50 factors (both are shown by semicircles). This conundrumcan be resolved by three alternative paradigms: large, static, multimeric complexes with dense packing; heterogeneous subpopulations that are bound by different subsets of factors; or dynamic sequential binding models that envision multiple transient states. Pink nucleosomes represent regions of chromatin that are undergoing dynamic reorganization, which results in accessible or ‘open’ chromatin. **b** | A dynamic view of enhancer functionis shown. The association between transcription factorsand regulatory elements is usually detected by ChIP–seq analyses across large cell populations. These cell groupsare highly heterogeneous with respect to local chromatin states and factor occupancy; variation is indicated schematically in the cell population by different colours. For brevity, four putative binding factors are depicted at a generic enhancer and their ChIP–seq signals are shown, although many more are often detected as co-binding. In static models, it is assumed that these factors form long-lived, multiprotein complexes. However, many dynamic processes within

individual cells contribute to the population-averaged signal. Multiple chromatin remodelling complexes[62] are probably recruited by transcription factors (which are colour coded to the ChIP– seq signal) to these elements; it is not known whether these processes are linear and sequential or whether they are cyclical. As cell division occurs, the population average occupancy, as well as participation in the underlying dynamic events (that is, assisted loading (FIG. 1)), evolves. A common assumption is that static, long-lived enhancer states that are observed across the cell population interact over long ranges to target promoters as shown by the dashed arrows. In fact, the chromatin states with brief lifetimes that contribute to the population average may separately communicate with promoters on a much shorter timescale. **c** | The dynamic model predicts many intermediate states. Some states may have lifetimes that are sufficient to produce factor footprints in cell population experiments, whereas others are probably too short-livedto be detected, even by formaldehyde-based ChIP.

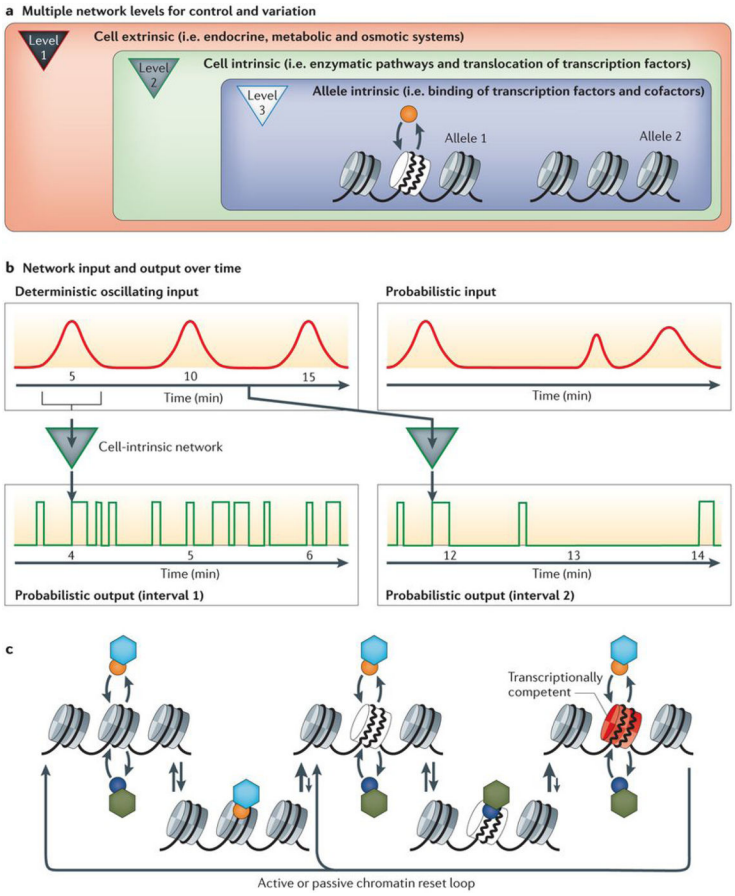

Figure 4. Multiple levels of dynamic organization control chromatin state and transcriptional output over time.

a | Dynamic events produce different temporal response patterns. Some of these responses — which range from intracellular signalling to transcriptional control — are highly reproducible and seem to be deterministic, whereas other responses are much more random and/or probabilistic in nature. Signalling network loops operate at the level of the organism (level 1), the individual cell (level 2) and the individual allele (level 3). Depending on specific configurations, these multiple levels can interact with one another to either increase or decrease the ordered activation of transcription. **b** | The top left panel shows a deterministic oscillating temporal signal that regulates a cell-intrinsic transcriptional control system. This stochastic signalling network may respond to these inputs either by increasing frequency of output signals during stimulation (bottom left panel) or by decreasing frequency in the absence of stimulation (bottom right panel). The top right panel indicates that the oscillating signal may be probabilistic rather than deterministic, which introduces further stochasticity in the system. **c** |The ‘hit-and-run’ transcriptional control model involves dynamic protein–protein interactions between transcription factors (hexagons) and co-regulators (circles), as well as protein–DNA interactions between these proteins and their target DNA. This stochastic process produces a chromatin template that cycles through multiple states, including a transcriptionally competent state (shown in red). This process may produce a deterministic linear series, or it may be partially reversible. The output of

these dynamic states integrated over time produces transcriptional activation events that seem to be deterministic at the averaged whole-cell population level.

Table 1

Techniques for assaying dynamic processes on chromatin

Method	Time resolution	Advantages	Disadvantages
Cell population methods			
ChIP-seq[115]	Minutes to hours	• Crosslinks can be easily reversed • Technology is well-established	• All population methods are limited by the cell-to-cell synchrony of the process • Formaldehyde produces caging effect • Detects tethered proteins that are not bound to DNA
Competition ChIP[116]	20 minutes	Standard approaches are accessible to many laboratories	• Insensitive to rapid exchange • Dependent on strong ChIP signal
UV laser crosslinking[117,118]	Milliseconds to seconds	Directly detects DNA-protein interactions	• No easy method to reverse crosslinks • Damage in DNA inhibits amplification of DNA
DMS footprinting[119]	Minutes to hours	Base-pair resolution can be achieved	Detection is highly factor specific
Single-cell methods			
Photobleaching (such as FRAP[120], FLIP[121], FLIM[122] and photoactivated GFP)	Milliseconds to seconds	Measures real-time movement in living cells	• Computationally complex • Total nuclear measurements average multiple interactions • Requires amplified gene arrays to observe specific binding
Spectroscopic methods (such as FCS.RICS[112], and number and brightness correlation spectroscopy[113])	Milliseconds to seconds	• Measures real-time movement in living cells • Detects true molecular interactions	• Requires complex instrumentation • Computationally complex
Single-molecule tracking[123]	Milliseconds to seconds	Directly observes molecular movement	• Nature of interaction target may be ambiguous • Requires labelling with bright chromophore
Chromatin reconstituted in vitro			
UV laser crosslinking[118]	Milliseconds to seconds	• Measures real-time interactions • Directly detects DNA-protein interactions	Requires complex instrumentation
Chemical crosslinking	Seconds to minutes	Measures real-time interactions	No standard method for choosing crosslinking reagent
Single-molecule methods[124]	Seconds	• Unambiguously identify interactions	Requires complex instrumentation

Method	Time resolution	Advantages	Disadvantages
		• Molecular-level resolution can be achieved	

ChIP–seq, chromatin immunoprecipitation followed by sequencing; DMS, dimethyl sulphoxide; FCS, fluorescence correlation spectroscopy; FLIM, fluorescence lifetime imaging; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; RICS, Raster image correlation spectroscopy; UV, ultraviolet.