MODES OF TRANSCRIPTIONAL REGULATION

Eukaryotic transcriptional dynamics: from single molecules to cell populations

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Abstract | Transcriptional regulation is achieved through combinatorial interactions between regulatory elements in the human genome and a vast range of factors that modulate the recruitment and activity of RNA polymerase. Experimental approaches for studying transcription *in vivo* now extend from single-molecule techniques to genome-wide measurements. Parallel to these developments is the need for testable quantitative and predictive models for understanding gene regulation. These conceptual models must also provide insight into the dynamics of transcription and the variability that is observed at the single-cell level. In this Review, we discuss recent results on transcriptional regulation and also the models those results engender. We show how a non-equilibrium description informs our view of transcription by explicitly considering time- and energy-dependence at the molecular level.

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Transcriptional regulation in the nucleus is the culmination of the actions of a diverse range of factors, such as transcription factors, chromatin remodellers, polymerases, helicases, topoisomerases, kinases, chaperones, proteasomes, acetyltransferases, deacetylases and methyltransferases. Determining how these molecules work in concert in the eukaryotic nucleus to regulate genes remains a central challenge in molecular biology. Dynamics lie at the heart of this mystery. Megadalton complexes assemble and disassemble on genes within seconds[1,2]; nucleosome turnover ranges from minutes to hours[3]; and gene activity demonstrates complex temporal patterns such as oscillation and transcriptional bursting[4,5]. Exciting new experimental advances have enabled the study of dynamic transcriptional regulation at the single-molecule[6] and genome-wide[7] levels, thus enhancing our understanding of transcriptional regulation *in vivo*. These approaches also necessitate new models for describing gene expression. In this Review, we discuss recent *in vivo* results and the quantitative models that are motivated by those results.

Chromatin immunoprecipitation (ChIP) provides genome-wide occupancy profiles for chromatin-interacting factors at near base-pair resolution in populations of cells[8,9]. Using this approach on a genome-wide level has generated comprehensive maps of regulation on a gene-by-gene basis[7,8,10]. This population approach has been complemented by single-cell imaging techniques. Almost all factors that have been studied by live-cell microscopy exhibit dwell times on chromatin on the order of seconds[11], and single-cell studies demonstrate a great variability in gene expression among cells in a population, owing in part to the stochastic nature of transcription[12]. Despite these tremendous advances in understanding the behaviour of individual factors, both methods fall short of capturing the sequence of events that is required to activate or repress a gene *in vivo*. Ideally, the occupancy of many factors that are coincident on a single stretch of DNA would be measured to obtain a sense of the complexes and intermediates that assemble *in vivo*. However, this experimental challenge is a daunting one. Current re-ChIP (also known as sequential ChIP) experiments usually look at two factors[4,13] but it would be necessary to look at an order of magnitude more factors to begin to capture the combinatorial complexity of transcriptional regulation in metazoans[4,14–16].

The gulf between actual mechanisms of transcriptional regulation and experimental capabilities could be bridged by using quantitative models of transcription. Decades of biochemical, structural and genetic data have spawned multiple models of transcriptional regulation, several of which we discuss below (FIG. 1). Even though these views are not mutually exclusive and boundaries between them are not clear, they reflect fundamental

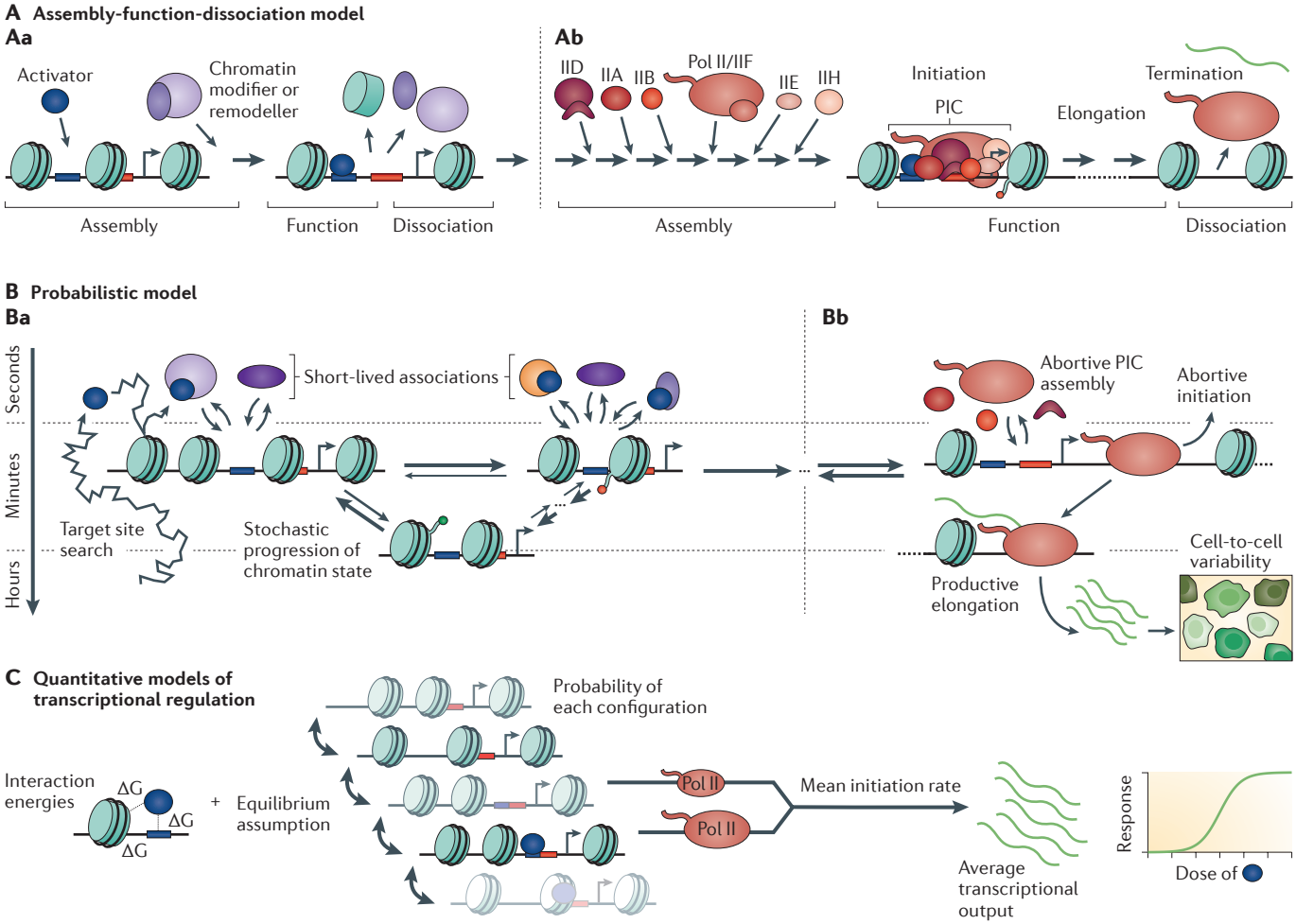


Figure 1 | **Points of view on transcriptional regulation.** Three schemes are shown that emphasize different aspects of transcription and gene regulation. Although these descriptions are not mutually exclusive, each scheme results in a particular bias regarding the temporal behaviour of single genes. **A** | The assembly–function–dissociation model. Experimental approaches based mostly on *in vitro* reconstitution or bulk, population-level assays aim to determine the molecular players that are involved at different stages in the transcription process. These methods clearly show that transcriptional activation is the result of a series of events that occur in a certain sequence. However, this scheme tends to describe the recruitment of complexes as a rather static and deterministic process. The example shown is of transcription initiation: **Aa** shows the uncovering of binding sites for the core machinery and **Ab** shows ordered assembly of the pre-initiation complex (PIC). **B** | The probabilistic model, showing the same stages of transcription as in panel **A**. Experiments based primarily on fluorescence microscopy can address questions relating to the kinetic aspects of transcription over various timescales. Such experiments have revealed that interaction times vary substantially but are generally short for most regulatory molecules. They show various slow temporal patterns in the transcriptional responses, with a substantial level of cell-to-cell variability. Coloured shapes represent factors that interact with chromatin (represented by a stretch of nucleosomal DNA). Curved arrows represent short-lived associations; arrows of different weights represent reversible reactions in which the forward and reverse reactions have different probabilities. **C** | Quantitative models of transcriptional regulation. Computational methods have been developed to quantitatively relate the concentration of regulators to average transcriptional activity, based on protein–DNA and protein–protein interactions. These models generally do not explicitly consider the intrinsic dynamics of the processes involved. In the example shown, the interaction energies between a factor, a binding site and a nucleosome (left) are used to compute the probabilities of different configurations of the regulatory region of the gene (centre). Given the different rates of transcription initiation that each configuration would lead to (represented by different sized polymerases), this approach can relate the concentration of the regulator to the transcriptional output of the gene (shown in the graph on the right). IID, transcription factor IID; Pol II, RNA polymerase II.

differences regarding the mechanisms of the underlying molecular processes. Currently, most quantitative theoretical models describe transcriptional regulation as an equilibrium thermodynamic phenomenon — an assumption that allows model building without explicitly considering the dynamics. Here we explain how this description is fundamentally inconsistent with the canonical view of gene regulation based on a sequential, ordered recruitment of factors, which is an example of a non-equilibrium model. In the context of a non-equilibrium model, the transcriptional dynamics can exhibit a form of molecular memory so that the future behaviour of the system depends on its history. We will outline this gap between the molecular biologist's canonical view of

transcription and the quantitative approaches that are often used to describe it. We argue for a non-equilibrium view of transcriptional regulation that is informed and constrained by single-cell observations. With the ability to observe single transcription factors[17] and single transcribing genes[18] in living cells, new experimental and modelling possibilities are emerging for understanding transcription dynamics *in vivo*.

The assembly–function–dissociation model

Data from *in vitro* reconstitution and population assays are often interpreted in terms of sequential molecular events. In a typical example of inducible RNA polymerase II (Pol II) transcriptional activation (FIG. 1A), an activator molecule first binds to a recognition site upstream of the transcription start site (TSS) and then recruits co-activators and chromatin remodelling machinery that eventually leads to nucleosome displacement at the core promoter, uncovering binding sites for the core machinery[19–21]. Thereafter, the pre-initiation complex (PIC) can assemble in an ordered manner[13,22–24]. Following several steps, including covalent modification of the Pol II carboxy-terminal domain (CTD), transcription can commence[25].

The essential idea to retain from these experimental results is that the events involved in transcriptional regulation seem to be intrinsically ordered (FIG. 1A). Although several alternative pathways may exist to reach the same outcome[26,27], it is clear that certain molecules cannot be recruited before certain steps have taken place. In this view, a given complex is recruited at the relevant stage, carries out its function (thus allowing the next step to occur) and eventually dissociates. Hence, even though kinetics per se are generally not of paramount concern in this scheme, the arrow of time occupies a central place. Moreover, a rate-limiting step may be invoked as the slowest molecular step in the process, which ultimately limits the overall progression and may therefore regulate the rate of transcription[19]. These notions of sequential recruitment and rate-limiting steps reflect a form of implicit kinetics, although it is usually considered in a steady-state, population-averaged context. These implicit kinetics will become explicit when we consider the case of stochastic transcriptional dynamics of single genes.

To address this notion of sequential recruitment, numerous population-level experiments may have an explicit temporal dimension. Typically, reverse-transcription PCR (RT-PCR) or ChIP is carried out over time after the induction of a gene to follow its transcriptional activity and promoter occupancy by various factors within a population of cells or chromatin templates[4,14–16,28–32] (FIG. 2A). For example, after the addition of oestrogen there is sequential recruitment to the trefoil factor 1 (*TFF1*) promoter of oestrogen receptor-α (ERα) and other factors including histone methyltransferases, histone acetyltransferases, then general transcription factors, and finally Pol II[4,14,15]. This recruitment process repeats for multiple cycles. Thus, ChIP-seq for ERα and other transcription factors that are associated with oestrogen-regulated transcription[33–37] has helped to define the concept of the oestrogen receptor 'enhanceosome', which is composed of ERα, GATA3, and forkhead box protein A1 (FOXA1)[38]. Although there is some debate about which factor arrives first (the so-called 'pioneering factor')[37–40], the essential underpinning of this description is that somehow the steroid-receptor ERα and the sequence-specific activators GATA3 and FOXA1 assemble sequentially on DNA to activate or repress oestrogen-regulated genes.

The corollary to this interpretation of population assays is that the requirement for multiple factors must reflect stable and long-lasting binding of molecules as part of a protein–DNA complex[41–44]. Indeed, it is often believed that complexes remain bound as long as they are observed on DNA in a ChIP assay and dissociate only when no longer needed. In this general view, the recruitment of different molecular partners progressively stabilizes the structure and facilitates the recruitment of other factors in a static and well-ordered manner.

Experiments in living cells complicate this view. For example, observations of ERα binding to DNA in single cells using a fluorescence microscopy assay showed that ERα is only bound to DNA on average for a few seconds[45], a period that is vastly shorter than the measured persistence of the ChIP signal. These data suggest that the peaks observed in ChIP experiments are static snapshots of dynamic processes that may only be occurring in a subset of cells. Moreover, average occupancy of a binding site at a promoter by a transcription factor does not correlate nearly as well with expression levels as the dwell time of that factor[46]. These experiments and other live-cell measurements have motivated an alternative model: the 'probabilistic model'.

The probabilistic model

Live-cell fluorescence microscopy probes the kinetic aspects of transcription and transcriptional regulation across a broad range of timescales that are much shorter than those probed in most population studies (FIG. 1B). This method can resolve the diffusion and binding of transcription factors in the nucleus, the stochastic assembly of complexes at promoters and enhancers and the time course of the transcriptional output (the latter of which is often imaged on gene arrays (FIG. 2B))[47–50]. These experiments also motivate a probabilistic model: one that is based on the stochastic interactions between transcription factors and DNA and that can account for the timing of the downstream transcriptional output resulting from those interactions. Time is a central notion in this model and is approached in a much more explicit and quantitative way than in the assembly–function–dissociation model.

Experiments such as fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) have shown that many of the molecules that are involved in transcription have short residence times on DNA relative to the timescale over which transcription takes place. Although a consensus across the various methods has not completely been reached[51,52], the residence times of molecules on their target sites range from seconds to minutes: sequence-specific transcription factors (for example, MYC, p53 and glucocorticoid

Chromatin remodellers
Along with chromatin modifiers, these are complexes and enzymes that affect the status of chromatin, through conformational changes (such as nucleosome displacement or eviction, or histone replacement) or by depositing or removing covalent marks on histone tails. These processes are accompanied by the hydrolysis of coenzymes, releasing chemical energy.

Chromatin immunoprecipitation
(ChIP). A method to assess the occupancy at a given genomic locus by a particular factor. It is carried out by amplifying DNA fragments that have been crosslinked to the factor of interest and pulled down using an antibody.

Re-ChIP
A modification of the chromatin immunoprecipitation (ChIP) method. By successively using several antibodies, it allows an assessment of the co-occupancy of a locus by multiple factors.

Equilibrium
For a chemical system to be at equilibrium, every reaction must occur in both directions with equal probability (or rate). Hence, a system can reach a steady state without ever being at equilibrium.

Steady state
Refers to a system that does not evolve over time. This concept may apply to various descriptions such as a set of concentrations of molecular species or the probability distribution of a set of features among a population of cells (for example, nucleosome positions on a specific promoter).

Fluorescence recovery after photobleaching
(FRAP). An experimental microscopy method to assess the mobility of molecules in living cells. In FRAP, the rate at which fluorescently labelled molecules repopulate a region of the cell that has been photobleached reflects both their diffusion and binding to chromatin.

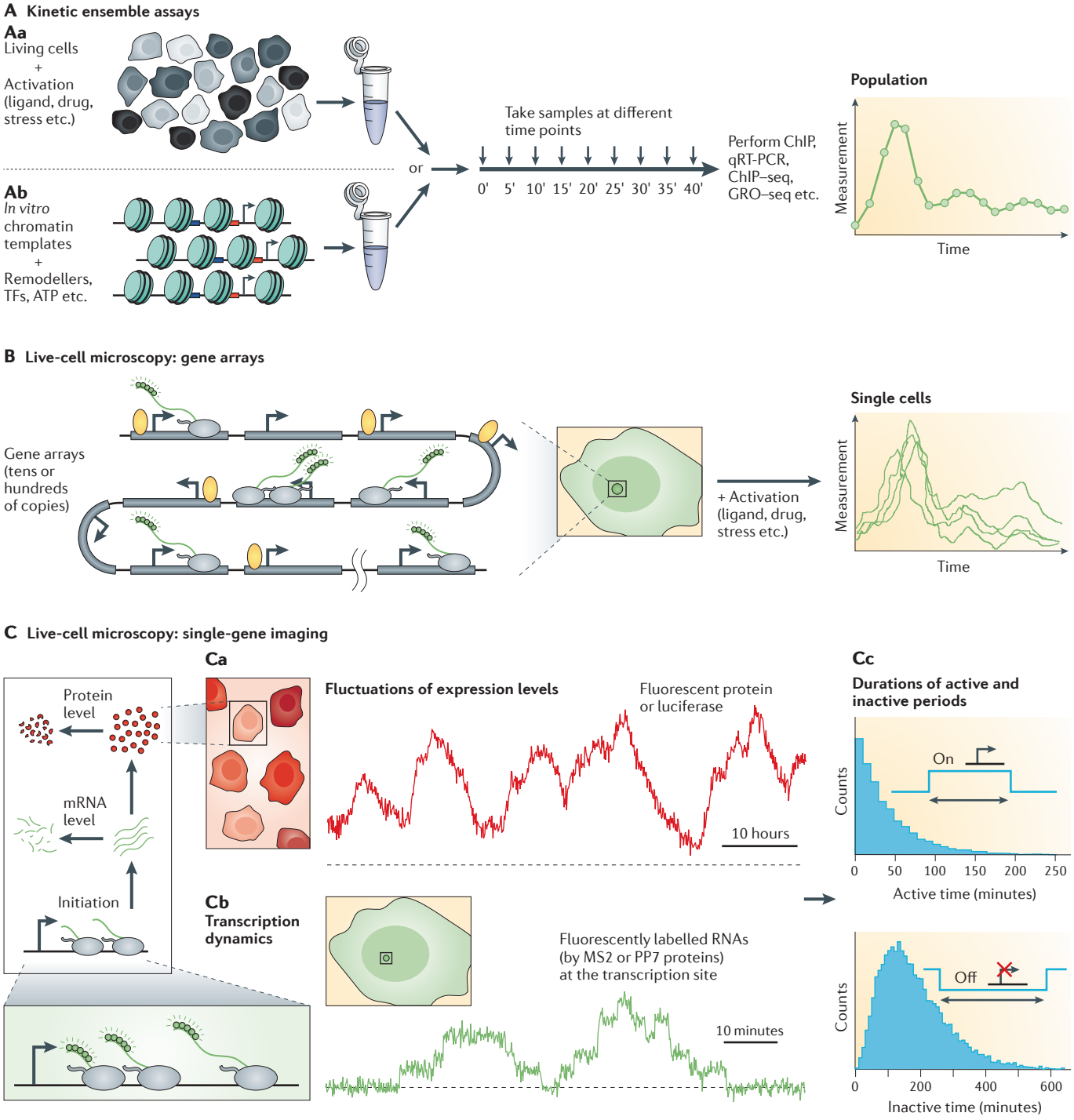


Figure 2 | **Experimental techniques to study transcriptional kinetics.** The dynamics of transcription can be probed using various techniques. **A** | Promoter occupancy and transcriptional output can be followed over time using bulk assays on either living cells (**Aa**) or reconstituted *in vitro* systems (**Ab**), thus yielding a population-level view of transcription dynamics. An example workflow for this type of experiment is shown. **B** | Live-cell imaging of gene arrays (gene copies are shown in grey) provides a single-cell picture, but this method still involves taking average readings across many copies of the gene of interest. Nascent RNAs visualized using MS2 and/or PP7 RNA labelling are shown in green. **C** | Single-gene dynamics can be probed either by following the fluctuations of fluorescence or luciferase activity of a reporter gene product (**Ca**) or directly at the transcription site, by monitoring the amount of nascent RNAs labelled with MS2 or PP7 fluorescent proteins(**Cb**). The resulting time traces can be interpreted using fluctuation correlation analysis[62] or hidden Markov methods[63] to reveal the kinetic scheme that the gene is following (for example, stochastic bursting or constitutive initiation). Single-gene methods also allow the estimation of parameters such as the initiation rate, the elongation time or (as shown in **Cc**) the distributions of time the gene remains in an active or inactive state. Data shown in **C** are simulated on the basis of schemes and parameters that are consistent with recent single-gene studies[18,62,63]. ChIP, chromatin immunoprecipitation; ChIP–seq, ChIP followed by high-throughput sequencing; GRO-seq, genomic nuclear run-on followed by high-throughput sequencing; qRT-PCR, quantitative reverse-transcription PCR; TFs, transcription factors.

receptor (GR)) are among the most dynamic, and the core histones (H2A, H2B, H3 and H4) are among the most stable[11]. Biochemical techniques have also been used to demonstrate turnover. For example, competition ChIP in yeast to study Rap1 and Gal4 indicates an average dwell time of ~60 minutes[46,53], and metabolic labelling of histones indicates a similar turnover rate[3,54].

Remarkably, the turnover kinetics of a given complex can vary depending on its engagement in the transcription process and can be subject to substantial modulations. For example, in specific conditions (such as a cell cycle phase, or during a response to stress or signalling), certain molecules can become more or less mobile, and therefore more or less likely to influence the probability of recruitment of other partners and/or to assemble a given complex. Examples of factors that have variable kinetics include transcription factors[45,53,55–57], core transcription machinery[58–60], chromatin remodelling complexes[61] and specific nucleosomes on the gene body and at the promoter[3,54].

Transient binding can result in patterns of transcriptional activity and gene expression that last many hours[62,63]. Indeed, single-cell analyses in various systems indicate that there is considerable variation (that is, 'noise') in gene expression[64], and this heterogeneity can be attributed in many cases to the stochastic nature of transcription, which often occurs in punctuated bursts of activity[5,6,65–68]. Understanding the connection between transient stochastic interactions and the slow temporal patterns of transcriptional activity has thus become a pressing question in the field.

The assembly–function–dissociation model and the probabilistic model are not necessarily mutually exclusive, but reflect a different mechanistic emphasis. In the assembly–function–dissociation model, the emphasis is on protein–DNA complexes that assemble in a particular sequence and are stabilized by cooperative interactions. In the probabilistic model, the emphasis is on fast, stochastic interactions between proteins and chromatin, and the implicit suggestion is that putative protein–DNA complexes represent a time-averaged view of occupancy rather than a stable assembly. Ultimately, these alternative concepts only have utility if they form the basis for quantitative, predictive models of transcriptional regulation, which is the subject of the next section.

Quantitative models of transcriptional regulation

Quantitative models have been developed to relate the concentration of regulators of a given gene to its transcriptional output (FIG. 1C). These models are commonly based on the interaction energies between molecules; that is, protein–DNA and protein–protein interactions[69]. In eukaryotes, these models have been quite successful at showing how nucleosome positions (both theoretically predicted and experimentally derived) at a promoter and their perturbation owing to binding of a transcription factor are usually highly predictive of the average transcriptional activity of a gene[70–72]. This approach can then be used quantitatively to derive a dose–response relationship between the concentration of the transcription factor and the expression of the gene (FIG. 1C). For example, the threshold for activation of the yeast *PHO5* promoter in response to inorganic phosphate is determined by the affinity of the transcription factor Pho4 for its cognate binding site, and the dynamic range of activation is controlled by the nucleosome occupancy of nearby sites[73]. Therefore, in this model the regulatory principle is based on the average occupancy of promoter DNA by different complexes, which subsequently influence the accessibility of specific sites such as activator sequences or the TATA box.

Equilibrium thermodynamics. The methods used in these models are based on equilibrium thermodynamics. The power of this formalism is that it provides steady-state properties of a dynamic process without having to consider the dynamics explicitly (BOX 1). Applied to transcriptional regulation, it allows the computation of the probability of each configuration of the promoter — resulting from the association and dissociation of molecules and displacement of nucleosomes on DNA — without considering the kinetic details (FIG. 1C). However, this formalism implies certain assumptions about the dynamics.

Although computational methods based on equilibrium thermodynamics have proven to be valuable for understanding the average transcriptional behaviour of a gene, they suggest an inappropriate picture when it comes to considering the temporal aspects of transcription. Crucially, the 'thermodynamic equilibrium assumption', which is central to these models, implies that no external energy is consumed (BOX 1). This approximation is well-acknowledged[72], but the result is that the energy dependence of chromatin remodelling and covalent modification reactions is hence considered to have only a marginal effect. This approximation is commonly justified by arguing that remodelling essentially serves to lower high energy barriers, thus helping the system to sample many configurations and hence to reach its equilibrium more quickly[72,74,75]. However, if the underlying kinetic details start to be considered, this approximation is clearly limiting.

As discussed in BOX 1, in an equilibrium steady state, each reaction is balanced; that is, it takes place equally in both directions. Hence, the simple idea of assembly followed by function and dissociation — for example, the fact that the chromatin is in a different state before a transcription factor, remodeller or modifier assembles and after it disassembles — cannot be captured by an equilibrium description. Indeed, it would require that any complex or enzyme that modifies or remodels the chromatin is able (and as likely) to do the exact reverse modification, even without having to dissociate and reassemble differently. On the contrary, complexes or enzymes that carry out opposite tasks are generally different (or have to bind the nucleosome in a different way) and are often recruited by different factors[76,77]. Although this phenomenology may only marginally affect the steady-state distribution of nucleosome configurations, it is very important when considering the time between two specific events, for example, the association and dissociation of a molecule (BOX 1C) or the inactivation of a gene and its reactivation (BOX 2).

Fluorescence correlation spectroscopy
(FCS). An experimental microscopy method to assess the mobility of molecules in living cells. In FCS, those properties are derived from the temporal fluctuations of fluorescence due to molecules entering and leaving a small optically defined volume of the cell.

MS2 and/or PP7 RNA labelling
A microscopy technique for labelling, in live cells, the transcripts from an artificial gene construct. Many molecules of fluorescent proteins (MS2 or PP7) bind each RNA on a specific cassette, thus allowing the monitoring of the number of nascent RNAs being transcribed at the gene locus over time.

Genomic nuclear run-on followed by high-throughput sequencing
(GRO-seq). This approach uses nuclear run-on methodology to map transcriptionally engaged polymerases on a genome-wide level. This approach constitutes a direct measure of transcriptional activity.

Box 1 | Equilibrium and non-equilibrium statistical thermodynamics

To describe the repartition of water over a landscape, it is possible to avoid referring to the complicated laws of hydrodynamics and instead to formulate the simple rule: 'water covers any location below a given altitude'. This description can accurately describe a lake, but not a river. Indeed, when water is actively displaced by evaporation and precipitation, it adopts a different repartition over the landscape (see the figure, part **A**). Such a system can still reach a steady state, but the rules to understand it are different: they require an explicit consideration of the dynamics.

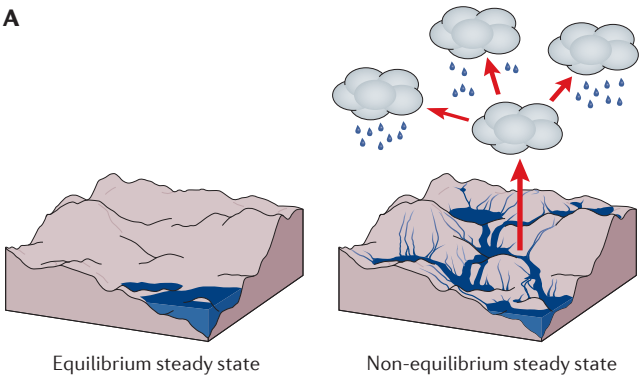
Energy consumption affects the way reactions are balanced

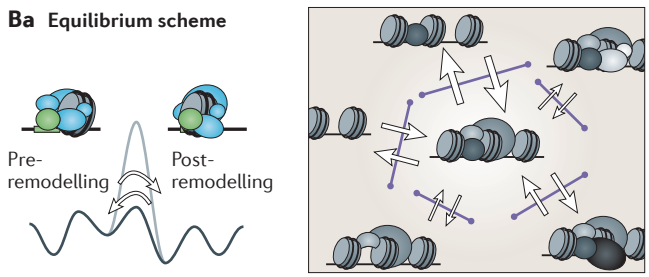
Equilibrium thermodynamics. The energy dependence of chromatin remodelling is often described as passively lowering activation energy barriers, thus facilitating reactions in both directions (see the figure, part **Ba**). This implies no actual energy transfer into the system and imposes a null net flux for every reaction (that is, all reactions occur equally in both directions; shown by equal-weight arrows). This is called 'detailed balance' or microscopic reversibility. This approximation allows the application of tools that avoid an explicit dynamic description.

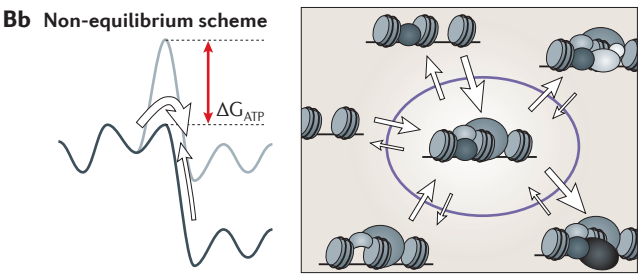
Non-equilibrium thermodynamics. Energy consumption actually facilitates a given reaction in only one direction (see the figure, part **Bb**), thus creating a driving force that maintains non-null fluxes (shown by unequal-weight arrows) throughout the entire system. Reaction rates only compensate globally for each state. This is called 'global balance'.

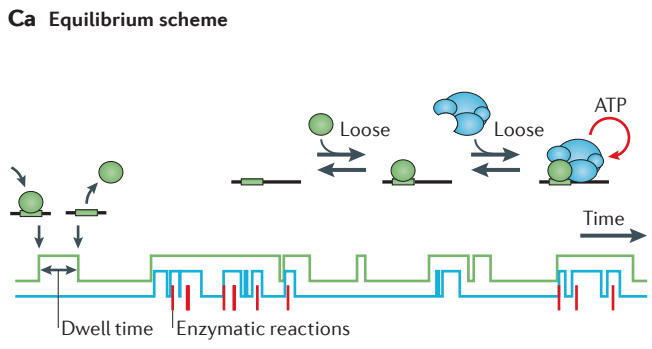
Fundamental kinetic differences

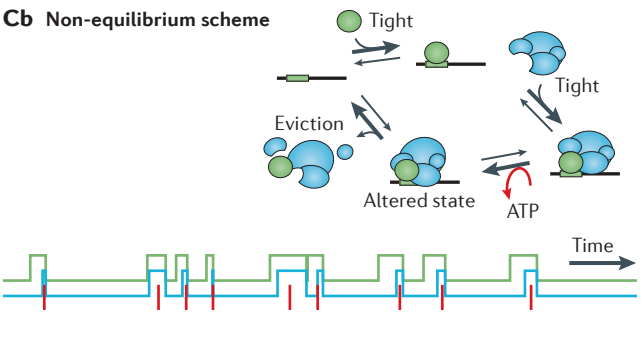
Part **C** of the figure shows that two biophysical models based on equilibrium and non-equilibrium schemes may have similar average ensemble-level behaviours (for example, occupancy levels, mean dwell times or the rate of the enzymatic reaction), but they differ in several fundamental kinetic aspects. For example, under the non-equilibrium scheme there can be both short dwell times and tight binding, the time distributions of dwell times can display refractoriness (the dwell time distribution peak is shifted to the right), and transcription factor (TF) binding has more-reliable outcomes (see the graphs of enzymatic reactions per transcription factor binding, where enzymatic reactions are shown as red vertical lines).

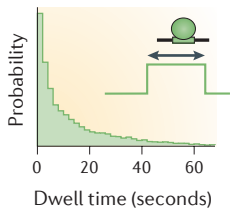


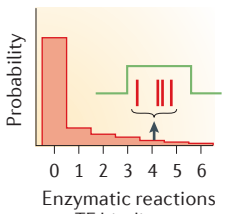


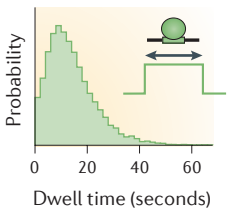


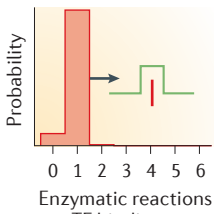












Non-equilibrium thermodynamics. A non-equilibrium model has several advantages for quantitatively predicting transcriptional behaviour. Experimental biologists have intuitively used non-equilibrium mechanistic descriptions of transcriptional regulation because this view considers directionality in reactions and cycling of molecular events (FIG. 1A,B). This directionality arises from a consideration of the energy dependence of reactions that are involved in transcriptional activation and repression[78–82]. The conceptual difference is that energy dependence may not just serve to make things happen more quickly or easily (as in the equilibrium view), but may give the dynamics a fundamentally different nature and make it follow different rules (that is, the non-equilibrium view) (BOX 1). The non-equilibrium view also takes into account how the kinetic organization of the molecular events at gene promoters has a crucial role in regulating the time course of transcription.

Equilibrium quantitative models are usually applied to pre-initiation processes (binding of transcription factors and displacement of nucleosomes), and non-equilibrium quantitative models are usually applied to transcription initiation and elongation[72,81,83–86]. We argue for a unified approach that is based on a non-equilibrium description, and give several examples of how this conceptual approach provides a different insight into transcriptional regulation.

The role of energy dependence

Differences between *in vitro* and *in vivo* experiments can often be illuminating. Here we describe two instructive examples of differing results from *in vitro* and *in vivo* experiments in which the key ingredient to reconcile the two is energy. Both cases reflect an inherently non-equilibrium view of *in vivo* dynamics.

Active nucleosome positioning. The position of nucleosomes on genomic DNA is markedly different *in vitro* and *in vivo*[87,88]. *In vivo,* an array of nucleosomes is well-positioned at the 5′ end of genes and nucleosomes are progressively less well-positioned further downstream in the gene body[89]. *In vitro*, only specific features — such as the nucleosome-depleted regions (NDRs) immediately upstream and downstream of the coding region — are reproduced[87]. Several thermodynamic equilibrium models have been proposed to explain this organization[90]. They invoke a combination of intrinsic DNA sequence preferences and exclusions[87,91] (for example, NDRs are partly due to stiff nucleosome-excluding DNA sequences) and a mechanism referred to as 'statistical positioning' by which closely packed nucleosome arrays form against NDRs owing to steric exclusion between neighbouring nucleosomes[92–94]. These models attempt to explain the difference between *in vivo* and *in vitro* data as being due to the nucleosome:DNA ratio.

However, two recent studies provide a different point of view. A first study showed that the inactivation of Pol II — a notable energy-dependent machine — forces *in vivo* positions to resemble *in vitro* ones[95]. Conversely, another study showed that the addition of ATP to a preparation containing reconstituted chromatin and whole-cell extract makes *in vitro* positions similar to *in vivo* ones, even at low nucleosome density[96]. Both of these studies suggest that energy-dependent machines, instead of simply facilitating nucleosome movements on DNA, actively drive and maintain the global nucleosome organization away from equilibrium (BOX 1). This example clearly illustrates how an equilibrium vision can be limiting in understanding even steady-state measurements.

Binding at regulatory sites: short and tight. A second example that illustrates the role of energy dependence when differentiating between *in vitro* and *in vivo* behaviour comes from studies of transcription factor binding. The short-lived associations of chromatin-interacting factors with DNA that have been reported using live-cell microscopy contrast strongly with *in vitro* measurements of protein–DNA complex half-lives on the order of tens of minutes to several hours[41–44]. One of the best studied examples is GR, which has a dwell time *in vivo* that is two orders of magnitude shorter than *in vitro*[43,97].

Part of the debate surrounding the idea of high turnover is the difficulty in conceiving how complexes can function properly if association is transient and hence viewed as unreliable and constantly subject to disruption. However, this outlook originates from an equilibrium view in which dissociation results from the thermal motion of the medium breaking contacts between molecules. Thus, the dissociation of a factor from its binding site reflects the interaction strength of the factor with its cognate site (which depends on the sequence specificity) and binding partners. In that context, the 'equilibrium constant' is defined as the ratio between the association and dissociation rates and is directly linked to the binding energy and the occupancy level of the binding site[98]. In this view, low occupancy levels and short residence times equate to weak interaction strengths. Although this principle may apply to the binding of a factor at nonspecific locations (that is, other than its target sites), it may not hold at regulatory sites.

Indeed, an essential aspect of the puzzle is that the mobility of molecules that is observed in living cells is closely related to various energy-dependent processes such as chaperone activity[56,99,100], proteasome-dependent degradation[45,56,100,101] and chromatin remodelling[55,102,103]. Thus, specific alterations of these processes or global depletion of ATP levels induce drastic changes in the mobility of the transcription factors in the nucleus. Active displacement of a transcription factor has also been shown *in vitro*, in a reconstituted system that recapitulates the interaction of GR with the mouse mammary tumour virus (MMTV) promoter template[29,55,104]. GR recruits the SWI/SNF chromatin remodeller and is evicted owing to the resulting energy-dependent remodelling. Importantly, this displacement requires the DNA template to be chromatinized and the presence of ATP. Without these, the transcription factor displays a high occupancy level at its cognate site, which is consistent with the stable associations that are classically observed *in vitro* in the

Flux
The net flux of a reaction is the difference between the rates at which it is observed to occur in one direction versus the other. When a system satisfies detailed balance, the net fluxes of all reactions are null.

Equilibrium constant
The ratio of association and dissociation rates. In an equilibrium context, this describes the affinity of a molecule for a binding site and directly relates to interaction energy. In a non-equilibrium context, such a ratio does not reflect the interaction energy and should not be called an equilibrium constant

absence of remodellers. Thus, the *in vitro* and *in vivo* data can be reconciled: when a factor binds a high-affinity site, its tight and reliable association leads to an energy-dependent reaction that eventually leads to its own eviction. The crucial consequence is that in the non-equilibrium context, transient interactions and low occupancy levels do not mean loose and unreliable associations (BOX 1Cb).

The main difference between the two schemes resides in the reason why a molecule dissociates. In the equilibrium scheme, the dissociation comes from thermal motion. In the non-equilibrium scheme, once a molecule is bound to a high-affinity site, it takes several kinetic steps before it dissociates (for example, recruitment of the energy-dependent cofactor followed by ATP hydrolysis). Hence, the distribution of time that the molecule spends on the binding site is fundamentally different and can display refractoriness (BOX 1Cb).

Moreover, this non-equilibrium scheme of 'short and tight' binding is more likely to provide reliable control of how the association and dissociation of transcription factors and cofactors affect downstream events. Indeed, as illustrated in BOX 1, the principle of a transcription factor being displaced by the enzymatic reaction it provokes implies that most associations result in one and only one enzymatic event. In a recent study of single-gene transcription kinetics in yeast[18], the measured rate of transcription initiation was in quantitative agreement with the expected rate at which a transcription factor encounters the promoter. Although not formally proven,

Box 2 | **Transcription timing reflects molecular processes**

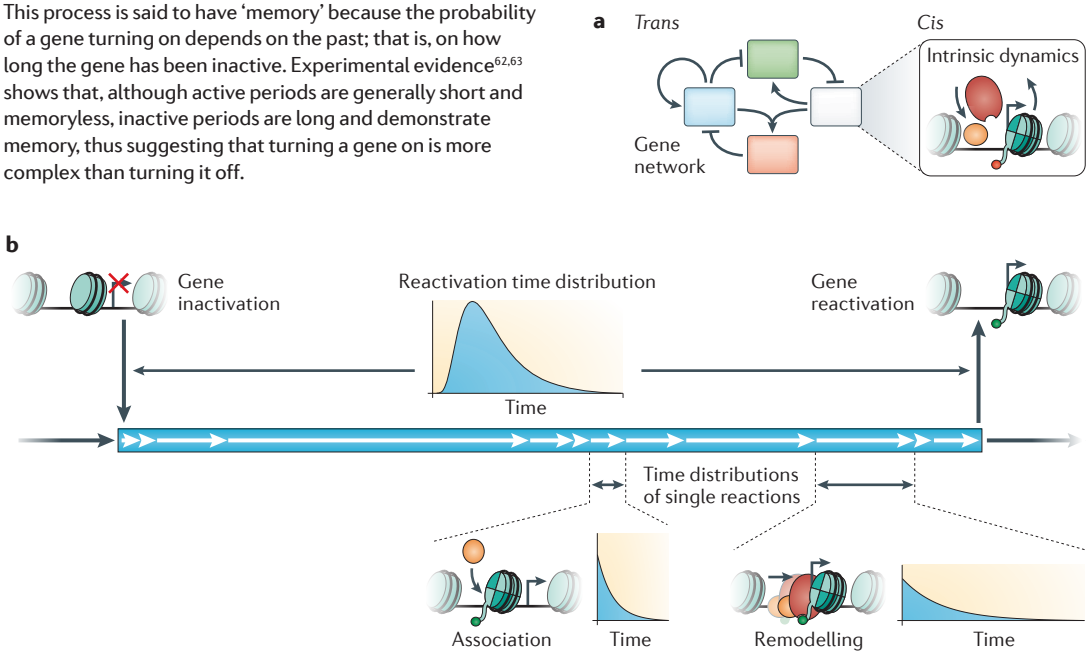
Cis and *trans* determinants in transcription dynamics

Complex temporal behaviours can emerge from gene regulatory networks. For example, a gene can display various types of oscillatory behaviours if the regulatory circuit in which it is involved provides negative feedback[151,152]. However, independently of fluctuations in *trans*-acting factors, a gene can also display intrinsic spontaneous dynamics stemming from the molecular events taking place in *cis* (for example, at the promoter or on the gene body) (part **a** of the figure). For example, an observation of uncorrelated oscillations of the activity of two reporter genes with identical promoters in single cells[62] demonstrated that periodicity can come purely from *cis* mechanisms. Another study[29] showed oscillations in a minimal *in vitro* reconstitution of glucocorticoid receptor (GR)-mediated SWI/SNF remodelling of chromatin templates; this assay lacked transcription, hence ruling out any regulatory circuit-based mechanism to explain oscillations.

Shaping the memory of transcription by multistep molecular processes

Recent live-cell microscopy experiments reported that the time it takes for a gene to reactivate after its inactivation can display a refractory period[62,63]; that is, the reactivation time distribution grows and then decays (part **b** of the figure). This property, which is at the origin of intrinsic *cis* oscillations, is a hallmark of non-equilibrium dynamics (BOX 1) and reflects the underlying molecular mechanisms.

The probability of a binding site being encountered by a cognate factor through random motion is independent of how long the site has been unoccupied; thus this is a 'memoryless' process and implies an exponential time distribution (see the 'association' graph in part **b** of the figure). The same applies to most elementary reactions, including dissociations, or enzymatic reactions occurring after the complex is assembled (see the 'remodelling' graph in part **b** of the figure). The distribution of reactivation time can only display refractoriness in a non-equilibrium context, in which the inactivation reaction does not occur equally in both directions and there are multiple molecular steps before a gene can turn back on. This process is said to have 'memory' because the probability of a gene turning on depends on the past; that is, on how long the gene has been inactive. Experimental evidence[62,63] shows that, although active periods are generally short and memoryless, inactive periods are long and demonstrate memory, thus suggesting that turning a gene on is more complex than turning it off.



Refractoriness
A time distribution function that displays an increasing phase for short delays is said to be refractory because — as opposed to an exponential distribution — the probability for the random event to occur is not constant but increases over time, thus shaping the distribution and reflecting the underlying biomolecular mechanics.

Memoryless
A process is memoryless if the time it takes to complete is exponentially distributed, indicating that its probability of completion does not change over time and is hence independent of the past. A succession of memoryless events (for example, sequential recruitment of factors) can lead to memory.

this nearly one-to-one ratio is consistent with the 'short and tight' binding mechanism.

The above hypothesis draws on elements of the probabilistic model and also on non-equilibrium thermodynamics. This view challenges the notion suggested by ChIP results that many factors simultaneously occupy the same piece of DNA. Rather, the combinatorial occupancy measured by ChIP may represent a time-averaged signal of factors that are present at regulatory elements such as enhancers[105]. To explore directly this hypothetical mechanism of transcription factor binding and action, it would be necessary to measure dwell times and ATP consumption at single active genes *in vivo*. Presently, such measurements are not available. However, it is precisely this gap that quantitative theoretical models are able to bridge.

Transcription kinetics

The dynamics of the transcriptional output provide a signature for the dynamics of the transcriptional input. In recent years, the ability to measure transcriptional output has improved dramatically, and here we discuss how time-resolved measurement of gene transcription from single-cell microscopy and ensemble biochemical assays can reveal underlying regulatory principles of gene expression.

Population-level measurements can provide substantial mechanistic information. Time-resolved ChIP and quantitative RT-PCR studies can elucidate the dynamic course of both the transcriptional response and upstream pre-initiation events[4,29,31] (FIG. 2A). Even steady-state measurements such as dose–response curves can reveal detailed mechanistic insight (BOX 3). However, for several decades we have known that the average behaviour of a cell population rarely reflects that of individual cells[64,106]. Indeed, transcription dynamics are closely intertwined with the variability in gene expression that is observed at the single-cell level (FIG. 2B). Often, the graded response of a cell population following treatment with increasing doses of an inducer does not reflect a progressive increase of the expression level of the induced gene in each cell, but rather stems from the increasing probability of a digital response in each cell[107]. Yet, this digital behaviour is not static in time[108]. In fact, recent time-lapse studies in single cells have solidified the notion that genes switch on and off with periods ranging from many minutes to many hours[18,62,63] (FIG. 2C). Thus, the digital response of a gene depends on when the cell is observed. Population measurements can be conceptualized as the superposition of many such dynamic events. Investigating transcriptional kinetics in both single cells and cell populations is therefore essential for understanding the range of behaviours that are exhibited in tissues[109] and even whole organisms[110].

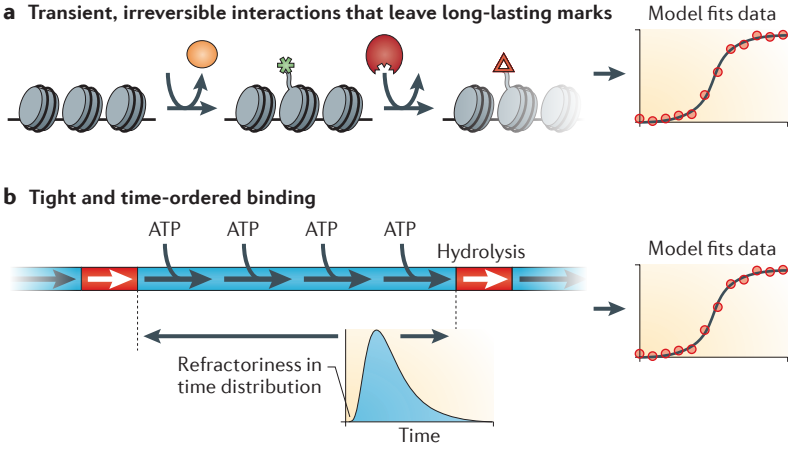
Dynamics at the single-gene level. The different sources of expression variability (that is, noise) in gene expression can be separated using various experimental and theoretical techniques[65,111–116]. In many cases, a substantial component of variability has been linked to a dynamic process taking place locally at the gene locus that is likely to involve the state of chromatin[65,68,112,115,117–119]. This process can be described as a probabilistic switch of the promoter between two states — an active state and an inactive state — thus defining time windows for transcription initiation events to take place. This model[120] can account for a continuous range of situations, from constitutive expression (that is, the initiation rate is constant over time) to brief pulses or bursts of transcription. In constitutive expression, polymerases fire individually; in bursting transcription, many polymerases are likely to fire in rapid succession (the active period), interspersed with periods of no polymerase firing (the inactive period). For example, in yeast, both kinetic modes of expression are observed: housekeeping genes display constitutive expression, whereas stress-response genes display bursting expression[18,121,122].

The fact that this temporal variability differs among genes and organisms (including bacteria and eukaryotes) suggests that transcription dynamics may reflect the underlying regulatory principles of a gene[123]. A first indirect approach to uncover these principles is to analyse the steady-state distribution of gene expression in a cell population. Examples of this approach include flow cytometry distributions of protein levels[68,117,124] or RNA counting in single cells using single-molecule fluorescence *in situ* hybridization (smFISH)[65,122,125,126]. Such studies have revealed that noise in gene expression strongly correlates with gene function and with promoter features such as the presence of specific DNA elements[122,124]. Chromosomal positioning of a gene also has a profound impact on the characteristics of its transcriptional bursting[68,115,117]. A second indirect approach to observe transcriptional dynamics consists of measuring the levels of a fluorescent reporter protein in single cells using time-lapse microscopy (FIG. 2Ca). From these data, the underlying RNA and promoter time-traces can be reconstructed using computational methods that can interpret the fluctuations in mechanistic terms[62,63,127]. For example, time-dependent fluctuations in a luciferase protein reporter were used to infer fluctuations in the transcriptional activity of the gene, and the bursting behaviour was linked to a *cis*-regulatory motif in the promoter[63]. Finally, a third and more direct way to observe transcriptional kinetics is to monitor nascent RNAs at the gene locus over time in live cells, using MS2 and/or PP7 RNA-labelling technologies[5,18,118,119,128,129] (FIG. 2Cb). Because this strategy relies on imaging RNA, it is possible to probe the relative contributions of initiation, elongation, termination and export to gene regulation[18,130–133]. In essence, these studies have revealed that any aspect of gene bursting can be regulated, including frequency[68,112,115,124,134,135], burst size[65,117,126,136–137], initiation rate during the burst[18,122] or some combination thereof[63,119].

Interpreting bursting kinetics. Single-cell time-lapse measurements now span a range from sub-second to days, thus providing unprecedented insight into the kinetics of transcription and possible clues to the upstream regulation. For example, two recent studies reported that genes exhibit a 'refractory period'

Single-molecule fluorescence *in situ* hybridization
(smFISH). A microscopy technique for labelling and visualizing RNA in fixed cells using many probes that are hybridized to a single transcript. For each cell, this technique allows the counting of the number of RNAs at the transcription site (that is, nascent RNAs) and also the number of RNAs in the cell.

Box 3 | **Dose–response and stochastic timing in a non-equilibrium context**



Steady-state population-level dose–response curves can harbour signatures of single-gene kinetics. Ong *et al.*[148] proposed a mathematical framework to decipher the order of recruitment of cofactors involved in steroid-responsive gene induction based on the shape of the dose-response curve[155]. They showed that, to account for the Hill coefficient value of 1 that was ubiquitously observed under various conditions, transcription factors and cofactors need to interact transiently with the gene template. This is consistent with a 'hit and run' scheme[50], in which cofactors only reside on chromatin for a short time, but leave a long-lasting modification that allows or facilitates subsequent steps (part **a** of the figure). Importantly, the model requires that template-modifying reactions cannot be reversed by the same cofactor — this is the essence of a non-equilibrium reaction scheme.

An instructive parallel is the functioning of a bacteriophage DNA packing ring ATPase motor. Using a combination of single-molecule optical manipulations and mathematical modelling, it was shown that DNA translocation occurs in this motor in rapid bursts that are separated by longer dwell times[140]. The distributions of these sub-second dwell times exhibit clear refractoriness, which stems from the multistep and sequential binding of four ATP molecules that precedes their hydrolysis (part **b** of the figure). Importantly, the rate of the whole process shows a dose dependence on ATP concentration that has a Hill coefficient of 1, which is contrary to what would be expected in an equilibrium context (the binding of four identical molecules would yield a steeper curve than the one observed). The authors showed using mathematical modelling that these two properties require: a tight and mostly irreversible binding of ATP molecules and that the four available docking sites on the motor recruit ATP in a coordinated, time-ordered manner (similar to the view in which gene reactivation requires an ordered sequence of events; BOX 2).

immediately following active transcription; that is, there is a minimal time that is required to reset a gene before transcription can begin again[62,63] (FIG. 2Cc). This kinetic feature reflects a 'memory' in the reactivation process[138] (BOX 2) stemming from the molecular events underlying gene activation. For a single biochemical step or, more likely, if one step is rate-limiting, there cannot be a refractory period. Indeed, this is the case when the gene turns off. Gene inactivation does not exhibit memory: the active gene has a given probability to inactivate, regardless of how long it has been active in the past. By contrast, a refractory period in reactivation reveals that several biochemical events are involved in shaping this delay[62,63] (BOX 2); that is, there are several biochemical reactions with rates in the same order of magnitude that are likely to contribute to the delay. In summary, these experimental data suggest that turning a gene off is simpler than turning it on.

Hill coefficient
A value describing the steepness of a dose–response curve at the level of transition between a low value and a high response. It reflects the level of cooperativity in the binding of the regulator and equals 1 in the case of uncooperative binding.

Importantly, memory in the reactivation process can only be understood in a non-equilibrium context; that is, when energy is consumed. Indeed, the multistep scheme that is used to explain the refractoriness of reactivation times implicitly relies on the presence of a 'ratchet'[62,63,79,82,138,139], which only moves in one direction. Without energy, reactions are equally probable in both directions, thus it is not possible to make a ratchet or to obtain refractoriness in time distributions (BOX 1). Interestingly, parallel phenomena occur in other molecular processes that operate at different timescales. For example, similar kinetic patterns have recently been observed in the functioning of a bacteriophage DNA packing motor at the single-molecule level[140] (BOX 3). How multiple elementary reactions shape such time distributions can be understood using queuing theory[141,142]. Thus, transcription time-profiles are likely to carry a substantial amount of information about the nature and organization of biochemical reactions at promoters (BOX 2).

The cyclic and sequential process we describe here for gene activation and inactivation needs to be carefully distinguished from the process known in the literature as the 'transcription cycle', although both have a multistep and cyclical nature. The 'transcription cycle' usually describes a polymerase-centred cycle consisting of initiation, elongation, termination and recycling of terminated polymerases for subsequent transcription[19,25,143]. This mechanism could in principle induce periodicities in transcription kinetics and might be detectable in microscopy experiments, but it cannot explain the bursting kinetics observed in mammalian cells: periodicities from polymerase recycling would have a period of roughly the elongation time, which is much shorter than the several hours of the bursting period[144]. Alternatively, polymerase recycling can be viewed as resulting in memory between single transcription events, whereas the memory that has been observed in single cells is between bursts of multiple transcription events[62].

Chromatin: the memory of transcription kinetics?

Although the molecular details underlying gene bursting have yet to be elucidated and might differ among genes, a proposed mechanism is that chromatin serves as the memory of the state of the gene, thus controlling the timing of activation and inactivation[50,139,145,146]. Because chromatin-interacting factors have rather short residence times, the progressive assembly of complexes seems to be an unlikely explanation to account for hour-long reactivation times[63]. By contrast, histones remain associated for much longer[11] and can carry metastable modifications. Hence, they constitute a reliable substrate for temporary marks that can serve as the basis for a slow multistep progression. This view reconciles fast upstream dynamics (of the binding of factors and cofactors) with slow downstream dynamics (of gene bursting) and provides a way to achieve a time-ordered recruitment of cofactors (BOX 3).

Hence, epigenetic modifications of chromatin not only can serve as a long-term memory through cell

Metastable
A biochemical or conformational feature can be qualified as metastable if it is very unlikely to disappear spontaneously without the intervention of an energy-dependent enzymatic reaction. For example, post-transcriptional modifications of histone tails are metastable, as are certain conformational or remodelled states of chromatin.

divisions — for example, encoding cell state or identity — but they can also have a central role in transcription kinetics. In neuroscience, working or immediate memory is described as "a limited capacity system, which temporarily maintains and stores information, […] providing an interface between perception, long-term memory and action."[147] Chromatin at promoter regions may be viewed as the working memory of transcription by being the substrate that is dynamically read and written both to integrate many incoming signals and to achieve temporal coordination among events that result in the production of RNA.

Relating single-gene and population assays. Can these single-cell dynamic signatures be observed in population studies? In some cases they can. Just as steady-state measurements such as dose–response curves can reveal non-equilibrium mechanistic principles[148] (BOX 3), time-resolved population experiments can reflect single-gene stochastic behaviour. Typically, these studies begin with the addition of an inducer: for example, after the addition of oestrogen, the various steps in transcriptional activation can be measured[4,36,149]. In the early stages after induction, the cells display a synchronous activation and are thus amenable to ensemble studies such as ChIP. Interestingly, this population response can reflect the kinetic signatures that are directly visible in studies of single genes in single cells. In particular, the refractoriness that is implied by a multistep process for transcriptional bursting is expected to result naturally in a periodic behaviour at the population level[78,79]. Many examples of periodic gene responses to activation have been published[4,14,16,28,29,32,66,150], and the progressive nature of cofactor recruitment that has been reported in these studies fits well with the multistep view of activation–inactivation that has been suggested by single-gene microscopy. However, oscillatory gene behaviour may also originate from *trans*-acting mechanisms, such as the interactions among several genes within a regulatory circuit[151,152].

Although evidence shows that purely intrinsic periodicity can exist (BOX 2), population-level oscillation can generally be expected to involve a mixed contribution of *cis* and *trans* effects. This point of view could help to understand population-level experiments more completely, such as the paradigmatic example of the *TFF1* gene response to oestrogen treatment[4]. In this context, although the observed spontaneous periodic expression behaviour of the gene is likely to originate from the ordered *cis* recruitment of protein factors, interactions among many genes undergoing a similar process could maintain synchronization and account for the stereotypical and regular nature of the observed time courses. Targeted induction of the gene of interest would minimize *trans* effects, but nonetheless a population measurement cannot separate the contribution of *cis* and *trans* mechanisms.

Outlook

The regulatory landscape of the human genome has come into sharper focus through the efforts of the Encyclopedia of DNA Elements (ENCODE) consortium, which has made tremendous strides towards identifying *cis*-regulatory motifs and the *trans*-acting factors that bind them[153]. A quantitative understanding of transcriptional regulation would allow those data to be coupled with proteomic data on transcription factor abundance[154] to predict the firing rate of a polymerase from a gene. Clearly, that aim is a distant one. Moreover, we also require that such a predictive approach should elucidate gene expression variation within a cell population, thus shedding light on subpopulations of cells that may be important for disease progression and development.

We have argued here for a model of transcriptional regulation that is based on both single-cell and population data that explicitly considers the role of time and energy consumption. This view provides an alternative conceptual paradigm for understanding combinatorial gene regulation and transcription dynamics.

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The authors declare no competing financial interests.