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Transcription in four dimensions: nuclear receptor-directed initiation of gene expression

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Regulated gene expression, achieved through the coordinated assembly of transcription factors, co-regulators and the basal transcription machinery on promoters, is an initial step in accomplishing cell specificity and homeostasis. Traditional models of transcriptional regulation tend to be static, although gene expression profiles change with time to adapt to developmental and environmental cues. Furthermore, biochemical and structural studies have determined that initiation of transcription progresses through a series of ordered events. By integrating time into the analysis of transcription, chromatin immunoprecipitation assays and live-cell imaging techniques have revealed the dynamic, cooperative, functionally redundant and cyclical nature of gene expression. In this review, we present a dynamic model of gene transcription that integrates data obtained by these two techniques.

Keywords: nuclear receptors; transcriptional cycling; ChIP; FRAP; model

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Introduction

The phenotypic diversity of cells, and their response and adaptation to their environment, are achieved through the regulation of gene transcription. Understanding how transcription is modulated is vital for describing the generation of cell-specific transcriptome and proteome profiles. Current models of transcription initiation tend to be static and centre on promoter elements that provide a platform for the assembly of intermediate complexes and the basal transcription machinery. The discoveries that transcription takes place in a repressive environment and that chromatin structure influences transcription further expanded these models (Ahmad & Henikoff, 2002; Brown, 1999). In parallel, a massively increasing number of co-regulators and protein complexes involved in transcription were identified (Belandia & Parker, 2003; McKenna & O'Malley, 2002; Narlikar *et al*, 2002), provoking the realization that functional redundancy is a generally applicable feature of transcriptional attainment. Together, these insights increased the complexity of transcriptional modulation and implied that the dynamics of recruitment are significant in gene expression. Moreover, the three-dimensional structure of transcription factors also has a significant impact on events (Asturias, 2004). Collectively, achieving transcription requires the integration of five variables: *cis*-acting factors (DNA and chromatin), *trans*-acting factors (transcriptional activators and associated complexes), the basal transcription machinery (including RNA polymerase II (Pol II) and TATA-binding protein (TBP)), three-dimensional structures and time. Additional hierarchical parameters, such as nuclear organization, also have an impact on these local events (van Driel *et al*, 2003).

Kinetic descriptions of transcriptional activation have been generated during the past five years by laboratories using nuclear receptor (NR)-driven gene expression as a model system. NRs are a subfamily of transcription factors, and include ligand-dependent transcription factors, such as receptors for oestrogens (ER-α and ER-β), androgens (ARs), glucocorticoids (GRs), progesterone (PR), thyroid hormones (TRs), vitamin D (VDR) and retinoids/rexinoids (RARs/RXRs; Robinson-Rechavi *et al*, 2003). Chromatin immunoprecipitation (ChIP) assays and fluorescence recovery after photobleaching (FRAP) have been used to evaluate transcriptional processes kinetically, albeit on different time-scales; ChIP has a time resolution of several minutes, whereas FRAP resolves events in the sub-second range. Essentially, each technique provides a different view of transcription, with ChIP indicating that, on average, transcriptional processes take tens of minutes and FRAP demonstrating a rapid exchange between transcription factors and their target promoters. In this review, we present a model that reconciles both data sets.

Chromatin and transcription

The organization of DNA into chromatin *in vivo* generates regulatory constraints that have central roles in many cellular processes (Khorasanizadeh, 2004). The basic organization of chromatin as a succession of nucleosomes separated by linker DNA is often likened to beads on a string. A nucleosome consists of 146 bp of DNA wrapped around histone octamers made of dimers of each of the core histones H2A, H2B, H3 and H4 (Luger *et al*, 1997). Chromatin structure has a dual influence on transcription: it organizes genomic information in three dimensions, which is important for the coordinated regulation of genome expression (Perkins *et al*, 2004), and it restricts

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the access of promoter sequences to the transcriptional machinery (Dillon & Festenstein, 2002). This structural restriction of chromatin on gene expression is overcome by multi-subunit protein complexes that have three main activities. First, reversible post-translational modifications (such as phosphorylation, acetylation, methylation, ubiquitylation and sumoylation) of the amino-terminal tails of the histones on lysine (K), arginine (R), serine (S) and threonine (T) residues modify chromatin structure. These alterations are directed by enzymes (for example, kinases, phosphatases, histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), ubiquitin and SUMO ligases) that associate with sequence-specific transcription factors binding directly to DNA (Gill, 2004; Narlikar *et al*, 2002). Specific sets of histone modifications are associated with genes that are actively transcribed and with those that are repressed. This defines the 'histone code' (Jenuwein & Allis, 2001), in which specific histone modifications imposed by one factor provoke the sequential recruitment of subsequent transcriptional factors. This adds further combinatorial and dynamic aspects to transcriptional regulation and increases the complexity of the information contained in chromatin: it is not only the sum of the charges on nucleosome tails that is important, but also their spatial combination and the order of their development. Second, plasticity of chromatin is induced by ATP-dependent remodelling complexes, which rearrange the organization of the nucleosomes in the chromatin fibre (Sif, 2004). Third, coupling CpG methylation by DNA methyltransferases (DNMTs) with deacetylation and methylation of histone lysines induces profound gene silencing and is typical in the organization of heterochromatin (Hermann *et al*, 2004). Finally, besides these enzymatic and energy-dependent processes, dynamic and competitive interactions of histone H1 and variants also modulate the local structure of the chromatin fibre (Catez *et al*, 2004; Bustin *et al*, 2005).

Regulation of transcription by nuclear receptors

NRs are transcription factors that bind as dimers to cognate response elements (PuGGTCA or PuG(G/A)ACA) that are organized in palindromic or direct repeats, or as monomers (Claessens & Gewirth, 2004). After binding to a promoter, NRs modulate transcription by recruiting transcriptional co-regulators and components of the basal transcription machinery. In the absence of ligand or, in the case of ER, when bound to partial antagonists such as tamoxifen (Lavinsky *et al*, 1998), NRs recruit repressive complexes to target promoters; these include HDACs, ATP-dependent remodelling complexes and corepressors such as SMRT and NCoR. These complexes generate a local chromatin environment that actively restricts transcription (Bowen *et al*, 2004). An exception to this are the 'classical' steroid receptors, such as GR, which, in the absence of ligand, reside in the cytoplasm. Binding of agonistic ligands induces the exchange of corepressors for coactivators through a structural rearrangement of the NR (Glass & Rosenfeld, 2000). So far, more than 100 cofactors of NRs have been identified, including ATP-dependent remodelling complexes such as SWI/SNF, complexes with HAT activities (such as the SRC/p160 family, CBP/p300 proteins and ADA complexes), and proteins with HMT activities (including CARM1 and PRMT1; Klinge, 2000; Lonard & O'Malley, 2005; McKenna & O'Malley, 2002). Another class of coactivators, known as TRAP–DRIP–mediator complexes, has also been identified (Fondell *et a*l, 1996); these interact with NRs at the identical surface to the p160/HAT proteins (Ren *et al*, 2000). Conceptually, recruitment of p160 and TRAP–DRIP complexes are mutually exclusive, as they cannot bind simultaneously to the same surface, which implies that they interact consecutively with the NR. Specific recruitment of the repressor of oestrogen receptor activity (REA), receptor-interacting protein 140 (RIP140) and ligand-dependent nuclear receptor corepressor (LCoR) by agonist-bound NR also displaces coactivators (Martini & Katzenellenbogen, 2003; White *et al*, 2004). Allosteric changes induced within interacting partners, such as ER-α and TBP (Warnmark *et al*, 2001), or NRs and HATs (Demarest *et al*, 2002) also define ordered and sequential interactions. Furthermore, HATs and HMTs modify target proteins other than histones (Wang C *et al*, 2001), with reciprocal post-translational modifications provoking sequential recruitment of protein complexes (Chen *et al*, 1999). Additional allosteric effects integrate chromatin remodelling with transcriptional regulation by NRs. For instance, binding of CBP to promoters is alleviated by H3 methylation (Wang H *et al*, 2001), and acetylation of H4 stabilizes recruitment of SWI/SNF (Hassan *et al*, 2001). Collectively, these data indicate that sequential, highly ordered processes define transcriptional events.

ChIP analysis of ER-α-mediated transcription

The most detailed ChIP-based analysis of the dynamic mechanisms involved in transcriptional initiation has been obtained for ER-α-mediated gene expression. The kinetics of association of ER-α and Pol II on four promoters—pS2/TFF1, cyclin D1, cathepsin D (CATD) and c-Myc—show a periodicity of 40–60 min (Shang *et al*, 2000; Reid *et al*, 2003; Liu & Bagchi, 2004; Park *et al*, 2005). By using α-amanitin to synchronize responsive promoters, a non-productive, cyclical interaction between the pS2 promoter and unliganded ER-α was shown (Reid *et al*, 2003; Métivier *et al*, 2003). Furthermore, three types of cycling occur in the presence of oestradiol (E2), namely an initial unproductive cycle that prepares the pS2 promoter for subsequent transcription, followed by two alternating, transcriptionally productive cycles (Fig 1; Métivier *et al*, 2003). Importantly, sequential immunoprecipitations of chromatin (Re-ChIP), which detect the simultaneous presence of two proteins on the same pool of promoters, have identified six ER-α-containing complexes in the presence of E2 (Métivier *et al*, 2003). In the cycles, a given enzymatic function (for example, HAT, HMT or HDAC activity) is provided by one of the alternative proteins in these complexes, such that combinations of functionally redundant enzyme complexes accomplish transcription by different routes.

Achieving transcription

The initial cycle in the presence of ligand and cycling of unliganded ER-α are similar in character. Both generate a chromatin environment that is permissive for transcription without attaining transcription itself (Reid *et al*, 2003). ER-α initiates the association of chromatin remodelling complexes, with SWI/SNF recruited by liganded ER-α, whereas, in the absence of E2, the complex responsible for the initial remodelling has not been identified. This relocates the nucleosome associated with the TATA box of the pS2 promoter, such that the TATA box lies outside the DNA occluded by the histone core. The additional recruitment of complexes that have HMT and HAT activities then defines a transcriptionally permissive promoter. The achievement of transcription during successive cycles is initiated by ER-α, which induces the sequential recruitment of intermediate transcription factors, then the basal transcription machinery, which in turn recruits and activates Pol II. After initiation, the sequential and ordered recruitment of factors

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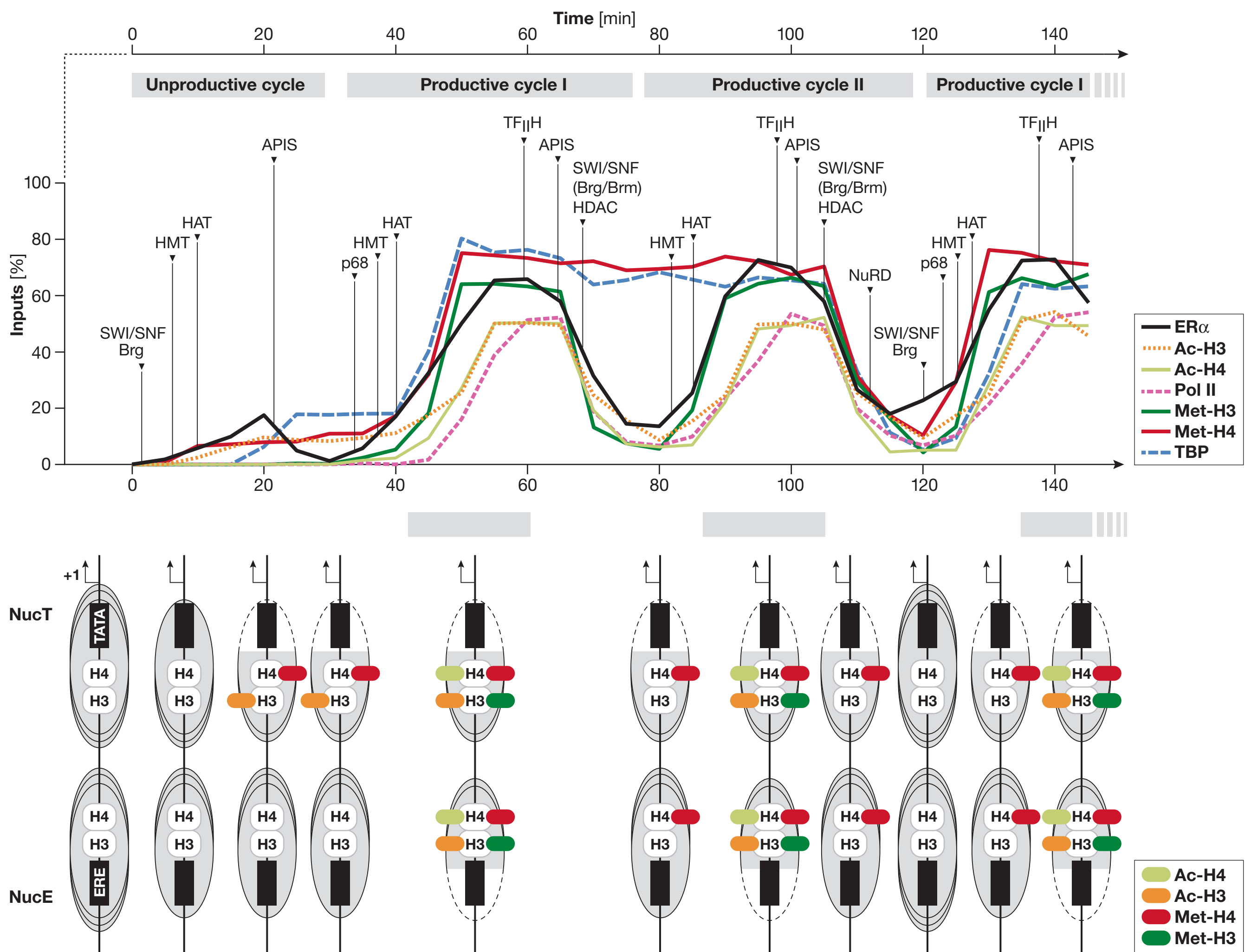

Fig 1 | Cyclical recruitment of transcription factors to the pS2 promoter. The recruitment of cofactors (top) and the dynamics of the nucleosome (bottom) mediated by oestrogen receptor-α (ER-α) on the pS2 promoter in MCF-7 cells in the presence of oestrogen. The periodic association of HATs, HDACs, HMTs and SWI/SNF (Brg/Brm), as well as other important complexes that contribute to ER-α dynamics and promoter clearance are shown with arrows. The association phase of each productive cycle is shown by grey bars. Location of the modified histones in nucleosome E (NucE) and nucleosome T (NucT) are shown, with increased accessibility of either the TATA box or the ERE shown by dashed lines. Schemes are based on Métivier *et al* (2003) and Reid *et al* (2003) and our unpublished data. Specific recruitment of NuRD at the end of the second transcriptionally productive cycle corresponds to NucT remodelling, displacement of TBP and demethylation of dimethylated H4 R3 (either complete or with only one CH_3 group). This step, which provokes the promoter to return to the basal state, delineates the two transcriptionally productive cycles. Ac-H3, acetylated histone 3 (K14); Ac-H4, acetylated histone 4 (K16); APIS, AAA ATPase proteins independent of 20S; ERE, oestrogen response element; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; Met-H3, dimethylated histone 3 (R17); Met-H4, dimethylated histone 4 (R3); NucE, nucleosome including the ERE; NucT, nucleosome including the TATA box; NuRD, nucleosome remodelling and deacetylating complex ; p68, p68 RNA helicase; TBP, TATA-binding protein.

defines the direction of cycling; transcriptional attainment is thus achieved through a transcriptional ratchet that ensures expression of the *pS2* gene. Unexpectedly, the presence of certain factors and post-translational modifications, such as the association of TBP and the dimethylation of arginine 3 (R3) in H4, persist over two cycles. Additionally, rearrangement of nucleosome phasing changes at the completion of every double cycle (Fig 1). These events reflect a sequential difference in the clearance phase of alternating transcriptionally productive cycles, in which complete resetting of chromatin organization correlates with removal of TBP. Physiologically, transcriptional cycling achieves the continuous sampling of oestradiol exposure and ensures an appropriate limitation to responsiveness.

Limiting transcription

Periodic limitation of transcription is generated by events that clear the pS2 promoter of transcription factors and induce a restrictive chromatin environment (Métivier *et al*, 2003). The proteins involved in resetting the pS2 promoter are generally implicated in transcriptional repression, but recent data have questioned whether this is

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their exclusive role (Ma, 2005). Activation of Pol II induces recruitment of 'repressive' complexes such as HDACs that direct the termination of each cycle. Three complexes act on the promoter to limit transcription (Fig 1). At the end of all productive cycles, HDACs, in association with the SWI/SNF complex, remodel local chromatin structure such that histone deacetylation restricts transcriptional engagement and the oestrogen response element (ERE) becomes associated with the nucleosome core. At the end of the second cycle, another ATP-dependent remodelling complex, NuRD, repositions the nucleosome associated with the TATA box, resulting in its occlusion and the exclusion of factors such as TBP. Illustrating the concept of the histone code, a transcriptionally engaged pS2 promoter can be defined by the presence of dimethylated H4 R3 and acetylated H3 K14. So far, the enzymes that achieve demethylation of H4 and H3 residues during transcription cycles of the pS2 promoter have not been identified. Candidates are PADI1/PAD4 and LSD1, which deiminate or demethylate the dimethylated residues, respectively (Shi *et al*, 2004; Wang *et al*, 2004). Rapid histone replacement through re-deposition, as shown during transcription elongation in yeast (Schwabish & Struhl, 2004), is an as-yet uninvestigated, alternative possibility.

The kinetically appropriate recruitment of E3 ligases and proteasomal components indicates that the degradation of the assembled transcriptionally active complex is also involved in the clearance phase of the pS2 promoter. Moreover, ubiquitylated proteins are found on the pS2 promoter, and inhibition of proteasome degradation abrogates transcription (Reid *et al*, 2003). Although ubiquitylated ER-α has never been detected on the pS2 promoter, and ER-α degradation and transcriptional activity can be dissociated in certain circumstances (Valley *et al*, 2005), proteasome function and transcription are inherently linked processes.

Transcription dynamics evaluated by live-cell imaging

Real-time, single live-cell imaging of transcription factors tagged with fluorescent proteins has also illustrated the dynamic nature of transcriptional activation, by showing that NRs are highly mobile in the nucleus (Hager *et al*, 2004; Maruvada *et al*, 2003; Rayasam *et al*, 2005; Schaaf & Cidlowski, 2003; Reid *et al*, 2003). Imaging of NR-directed transcription has been greatly facilitated by the use of tandem arrays of responsive promoters, which generate a high local concentration of responsive elements that are visible as a discrete locus when associated with labelled proteins (McNally *et al*, 2000; Tsukamoto *et al*, 2000). Fluorescence recovery after photobleaching (FRAP) analyses have shown that NRs and interacting cofactors rapidly exchange on these arrays (Becker *et al*, 2002; Stenoien *et al*, 2001a). This continuous sampling of responsive promoters suggests that transcription activation is achieved through stochastic mechanisms, generally known as 'hit-and-run' (McNally *et al*, 2000); consequently, stochastic, sequential initiation and limitation of transcription are predicted to result from the high mobility of transcription factors and other chromatin-associated proteins (Phair *et al*, 2004).

Reconciliating ChIP and FRAP data: a model

The apparent discrepancy between rapid events seen with live-cell imaging and longer cycling times determined by ChIP assays arises in part from the different time-scales examined in these experiments. This notwithstanding, the data obtained by ChIP and FRAP are robust. Whereas FRAP experiments mainly detect the bulk, rapid

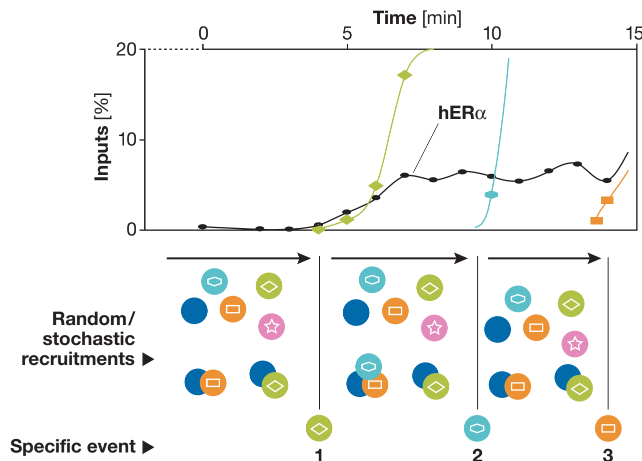

Fig 2 | Proposed allosteric, stochastic and dynamic model. This model integrates the general concepts that have emerged from detecting oestrogen receptor-α (ER-α) in live-cell imaging experiments such as fluorescence recovery after photobleaching (FRAP) and those from chromatin immunoprecipitation (ChIP) assays. This model incorporates both stochastic and deterministic concepts into transcriptional attainment. We postulate that transcriptionally productive complexes, which have slower mobility, are rarely formed on promoters. Transcription initiation requires a specific sequence of events to occur, defining a transcriptional ratchet that orientates progression through the cycles. Before that one deterministic event takes place, many rapid stochastic and transient associations of factors occur that are unproductive. It is only when a specific required factor is recruited at the appropriate time that progress is made. Allostery is instrumental in these transitions, as functional, three-dimensional changes are anticipated to occur on all participating partners (namely, proteins, DNA and RNA). Whereas FRAP experiments mainly detect the rapid, unproductive binding of factors, ChIP assays allow the determination of the precise kinetics of the productive associations and of the time required for the transition from one step to another.

and potentially transient binding of factors, ChIP assays only detect productive associations of promoter sequences with specific transcription factors. Taking into account these limitations, we propose a model (Fig 2) that integrates the high mobility of NR as observed by FRAP with the longer cycle times determined by ChIP.

We postulate that transcriptionally productive complexes have a slower mobility than transcription factors not engaged on a promoter. In general, and as determined by ChIP assays, initiation of transcription requires specific sequences of events to occur; these are ordered, kinetic and directional. These processes define a transcriptional ratchet that orientates progression through the cycles and is dependent on productive events that occur infrequently from many rapid, stochastic, transient and unproductive associations of factors. On average, many factors rapidly but non-productively associate with a promoter before a deterministic event takes place. Such continuous scanning is essential for transcription and is mirrored in the high mobility seen by FRAP. It is only when a specific and required factor becomes recruited at an appropriate time that progress is made. Allostery is instrumental in these transitions, as functional, three-dimensional changes are anticipated to occur on all participating partners (that is, proteins, DNA and RNA). Specific

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events that modulate chromatin also orientate the sequence of recruitment and act as a transcriptional ratchet that determines the direction of cycling. After promoter synchronization, kinetic ChIP evaluations determine the average time required for transition from one phase of transcription to another.

This model proposes that transcriptionally productive complexes, with slow mobility, are rarely formed on promoters. In accordance with these principles, functional engagement of a protein results in a restriction of mobility. By inference, the overall high mobility of NRs reflects a low probability that association with a promoter element is functionally productive. Interestingly, there are at least two kinetic components in the rate of recovery after photobleaching. Although some mathematical models indicate that, in some instances, a single-molecule population can generate biphasic FRAP curves (Sprague & McNally, 2005), other interpretations suggest that these components might reflect the existence of a rapid fraction, probably consisting of non-productive, freely diffusing and scanning NR, and a slower component that perhaps reflects productively engaged NR (Phair *et al*, 2004). In addition, this model implies that some events do not follow a stochastic binding process. For instance, histone modifications define a given promoter state; this acts to ensure the direction and progress between phases of the transcriptional cycle.

In accordance with this model, the interaction of GR with structural chromatin components, such as high mobility group box 1 (HMGB1), slows down the mobility of GR (Agresti *et al*, 2005). In each period of residence, a bound factor has to recruit another available and required protein. If this partner is not recruited, then the factor dissociates. If it is recruited, then the resulting complex becomes stabilized on the promoter, thereby advancing the cycle and precipitating the next event. Therefore, it should be possible to define the complexes that are present at each step of the cycle by their association and dissociation kinetics. Interestingly, this probabilistic, deterministic model is in accordance with data from Dundr and colleagues (Dundr *et al*, 2002), who showed that the assembly of the RNA polymerase I transcription complex proceeds in a sequential manner through metastable intermediates created through random 'collisions' (Vermeulen & Houtsmuller, 2002). Another inference of this model is the distinction of two 'clearance' mechanisms: one that is inherent to the stochastic recruitment and stimulation of the transcriptional machinery; and a second, cyclical 'active clearance' inherent to a promoter that has cyclical activity. This distinction is a kinetic distinction not related to the relative energy dependency of each of these mechanisms, as rapid cycling of at least some transcription factors requires ATP (Karpova *et al*, 2004; Stenoien *et al*, 2001b).

How is transcriptional cycling initiated? Potentially, transient dissociation of nucleosomes is sufficient to expose an NR-binding site and to provoke subsequent transcriptional regulation. However, it is difficult to envisage how spontaneous dissociation from nucleosomes can create short windows of binding opportunity to allow promoters from a cell population to act synchronously after their release from transcriptional blockade. Alternatively, active clearance allows synchrony, with each ChIP peak representing a mean of stochastic, asynchronous states. The kinetics seen by ChIP therefore reflects the delay required between each state. If spontaneous dissociation of nucleosomes is vital to initiate the system, this also questions the mechanisms of DNA-binding specificity and sequence recognition: are highly mobile NRs continuously scanning the entire genome for adequate binding sequences? ChIPs performed on specific arrays found that ER was associated with large regions of chromosomes 21 and 22, with some located outside E2-dependent gene sequences (Caroll *et al*, 2005; Laganière *et al*, 2005). This may be in accordance with a scanning process.

A model for NR-mediated transcriptional activation?

How general is the phenomenon of promoter cycling? In addition to ER-α, detailed kinetic ChIP analysis of the association of transcription factors with a cognate promoter have been reported for AR, TR and VDR (Kang *et al*, 2002; Sharma & Fondell, 2002; Vaisanen *et al*, 2005). In each case, and without promoter synchronization using α-amanitin treatment, cyclical recruitment of transcriptionally competent complexes has been observed with a periodicity of 50–80 min. In contrast to AR and ER-α, TR persists on responsive promoters (Sharma & Fondell, 2002) and represses target promoters in the absence of ligand, perhaps indicating that TR-dependent cycles are generated by sequential changes in the transactivation capacity of TR. Other specific mechanistic details of NR-mediated cyclical transcription also exist. For instance, inhibition of the proteasome stimulates GR activity, which is in direct contrast to ER, although both NRs become immobilized on proteasome inhibition (Reid *et al*, 2003; Wallace & Cidlowski, 2001). Furthermore, on the mouse mammary tumour virus promoter, proteasome activity is required for PR clearance, but it is chaperones that are involved in GR clearance (Freeman & Yamamoto, 2002; Dennis *et al*, 2005).

Although ER-α-mediated transcriptional cycles have been observed on four gene promoters, a more complete analysis evaluating different types of oestrogen-responsive promoters would address potential correlations between promoter structure, cycling periodicity and cofactor engagement. For example, the presence of many EREs affects ER-α transactivation capacity (Hall *et al*, 2002). TRAP/Mediator and p160 proteins associate simultaneously on the CATD promoter (Shang *et al*, 2000), in contrast to the situation found with the pS2 promoter (Métivier *et al*, 2003), which suggests a diversity in how individual promoters achieve transcription. Additional cognate binding sites for transcription factors on promoters have an impact on the sequence of recruitment. For instance, on the pS2 promoter, Sp proteins and AP1 influence ER-α activity (Barkhem *et al*, 2002; Sun *et al*, 2005). It is also probable that the association of the general transcription machinery on TATA$^-$ promoters might generate kinetics of association that are different to those depicted for the TATA$^+$ pS2 promoter. It is likely that additional specificity exists at the level of the recruitment of general transcription factors, depending on the architecture of the core promoter (Smale & Kadonaga, 2003).

Concluding remarks

It is evident that, for a limited proportion of promoters at least, transcription is attained by a cyclical progression of generating transcriptional competence, achieving transcription, then limiting this process through removal of the transcriptional machinery and resetting the histone code. Inherent to this progression is the concept of a transcriptional ratchet, in which the general use of post-translational modifications, acting as directional markers in time, orientates and progresses movement through the cycle. These new insights into transcriptional attainment and regulation

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provide new opportunities in understanding and influencing gene expression. They also offer new challenges, such as studying the kinetic interconnection between transcription initiation and splicing, and RNA maturation, which are processes regulated by NRs (Auboeuf *et al*, 2002). Finally, an outstanding issue will be to understand how to reconcile these highly dynamic models with other hierarchical regulatory elements of transcription, such as nucleus organization and recently identified transcription factories (Osborne *et al*, 2004).

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