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REVIEWS

Review

Transcription Factors and DNA Play Hide and Seek

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Transcription factors (TFs) bind to specific DNA motifs to regulate the expression of target genes. To reach their binding sites, TFs diffuse in 3D and perform local motions such as 1D sliding, hopping, or intersegmental transfer. TF–DNA interactions depend on multiple parameters, such as the chromatin environment, TF partitioning into distinct subcellular regions, and cooperativity with other DNA-binding proteins. In this review, how current understanding of the search process has initially been shaped by prokaryotic studies is discussed, as well as what is known about the parameters regulating TF search efficiency in the context of the complex eukaryotic chromatin landscape.

Highlights

Transcription factors (TFs) search their specific sites through both 3D diffusion and local motions.

TF search efficiency depends on TFs' biochemical properties and local concentrations.

Eukaryotic TFs vary strongly in their nonspecific DNA association and search efficiency.

Progress in temporal and spatial resolution of single-molecule microscopy will be required to better describe the nanoscale movements of TFs on chromatin.

Transcription Factors Are Universal Master Regulators of Gene Expression

Gene expression is central to all living systems and subject to a complex regulation all the way from RNA synthesis to protein degradation. Transcriptional initiation is the most important point of control in the gene expression cascade and is regulated by two classes of DNA-interacting proteins: (i) gene-nonspecific factors, such as RNA polymerase II, general transcription factors, and enzymes involved in histone modifications and DNA methylation; and (ii) gene-specific factors, also commonly called 'transcription factors' (TFs) [1]. Gene-nonspecific factors cannot by themselves determine which genes to regulate in a particular context. By contrast, TFs are characterized by their sequence specificity, which allows them to activate or repress transcription of target genes [1]. In prokaryotes, most TFs act as inhibitors of transcription by sterically hindering the progression of RNA polymerase. They do so by binding to a small number of genomic locations to regulate very specific aspects of bacterial life, such as lactose metabolism or biosynthesis of tryptophan [2]. By contrast, eukaryotic TFs have developed an arsenal of molecular mechanisms to deal with the complex biochemical environment of eukaryotic chromatin. This is achieved through specific domains allowing recruitment of non–sequence-specific regulators that impact transcription at different levels, such as DNA accessibility or recruitment of the RNA polymerase II machinery [1]. Despite very different modes of action, prokaryotic and eukaryotic TFs share the same fundamental challenge: finding their target sites among a vast space of nonspecific DNA sequences.

Finding Needles in a Haystack

TF molecules explore genomic DNA in a highly dynamic manner and occasionally reach specific stretches of nucleotides to which they bind with high affinity. The fraction of time that these DNA sequences are occupied by TFs is determined by two parameters: how often TF molecules land on these sites and how long the molecule stays bound. At equilibrium, this can be described by the law of mass action: $\frac{[TFb]}{[TF][BS]} = \frac{k_{on}}{k_{off}} = \text{kD}$, where $[BS]$ is the concentration of binding sites, $[TFb]$ is the concentration of TFs associated with binding sites, and k_{on} and k_{off} are the association and dissociation constants, respectively. The dwell time of TFs on specific sites is inversely proportional to k_{off}. Therefore, k_{off} is a metric for the binding strength between a TF and its specific site, while k_{on} reflects the efficiency with which a TF molecule finds a binding site in the genome.

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[*BS*] is determined by the genome sequence and the volume of the compartment in which it is contained. In eukaryotic cells, a large fraction of genomic DNA is wrapped around nucleosomes, and which regions remain nucleosome-free vary considerably across cell types. Therefore, the number of TF binding sites across different cell types of multicellular organisms is essentially invariant, but the sites accessible to TF binding can vary substantially [3].

When the number of specific sites and their accessibility to TF binding are constant, the frequency of binding events will depend on k_{on} and [TF]. The magnitude of k_{on} depends on the biochemical characteristics of TFs, which can be modulated by ligand binding or post-translational modifications in a generally on–off manner [4,5]. By contrast, [*TF*] can in principle be fine-tuned over several orders of magnitude [6–8]. To ensure efficient modulation of occupancy, TF concentrations have to be in a range that is comparable in magnitude to their dissociation constant, K_d [9,10]. Occupancy is expected to scale with [*TF*] linearly when specific sites are far from saturation. By contrast, the TF properties regulating k_{on} remain largely a mystery. Over 50 years ago, Adam and Delbrück formulated the idea that TF search for TFs' specific binding sites could be optimized by reducing the dimensions to be explored [11]. They proposed that instead of diffusing only in 3D, TFs could move along DNA in 1D by sliding, thereby increasing the number of sequences scanned per unit of time. These movements are driven by thermal fluctuations and may be impacted by collisions with other molecules bound to DNA. Thus, an exclusively 1D diffusion process may result in local trapping of TF molecules on segments of DNA representing only a small fraction of the genome. Therefore, TF search is an optimization problem, and the optimum was proposed to be reached at equal time spent in 3D and 1D diffusion regimes [12,13].

As discussed above, nonspecific DNA binding affects TF search efficiency and thus k_{on}, while specific DNA binding affinity impacts TF dwell time on specific sites (i.e., $\frac{1}{k_{off}}$). Nonspecific DNA binding is mainly mediated by electrostatic interactions with the negatively charged phosphate backbone of DNA. These depend on positively charged residues of TFs [12,14] such as nuclear localization signals, which can mediate sliding on DNA [15]. By contrast, TF binding to specific DNA sequences mainly depends on hydrogen bonding and van der Waals interactions between TF DNA-binding domains and specific bases on DNA [16], even though electrostatic interactions also play a role in stabilizing specific interactions [12]. Therefore, specific and nonspecific DNA binding can in principle be tuned at least partially independently. Nonspecific DNA interactions will govern the speed and length of TF sliding on DNA, which have nontrivial optima. Fast sliding ensures the scanning of a large number of sequences per unit of time, but it reduces the ability of TFs to stop and engage with specific binding sites [13]. The ability to switch to a specifically bound state also implies a change in the conformational state of the TF [14]. Finally, the sliding length depends not only on sliding speed but also on the average length of naked DNA segments, which is very different between the relatively naked prokaryotic DNA and the largely nucleosomal eukaryotic chromatin. In summary, a large number of parameters are predicted to govern search efficiency, and their optimum will vary considerably, depending on the chromatin context.

Searching the Prokaryotic Genome

Current understanding of how TFs search the genome *in vivo* has been strongly driven by *Escherichia coli* studies. In prokaryotes, the genome is organized in 3D by architectural proteins that bind to genomic sequences with loose specificity to bridge together different DNA segments (Figure 1) [17]. Despite some restrictions due to this organization, most bacterial promoters are accessible by default to the activity of RNA polymerase. Prokaryotic TFs act by directly binding to specific DNA sequences that are generally located close and 5′ to gene transcriptional start sites. This results in either recruiting RNA polymerase or impeding its progression. Prokaryotic

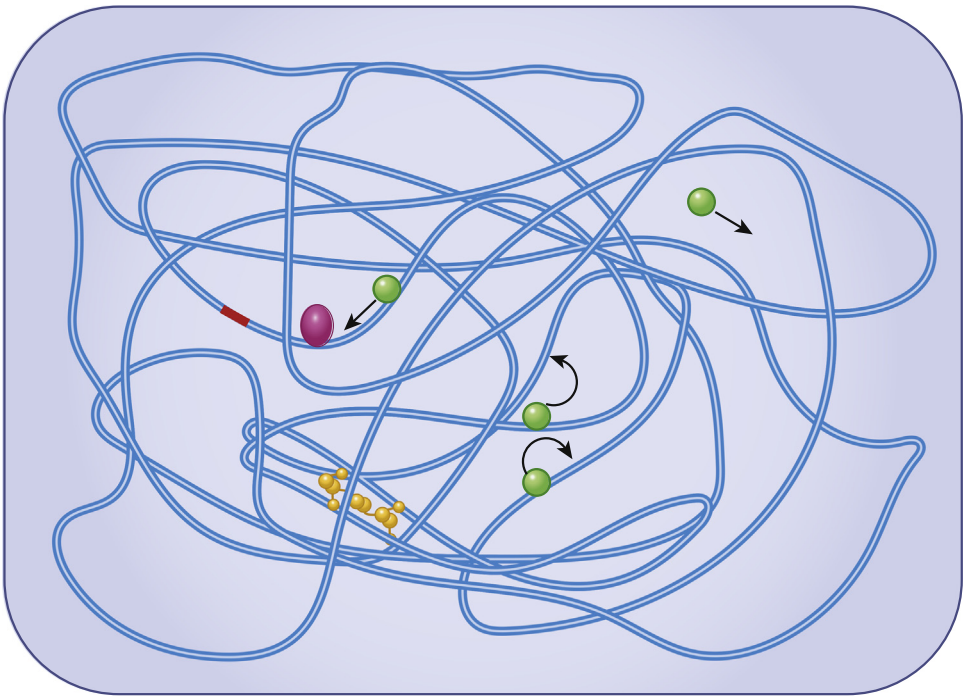

Figure 1. Transcription Factor (TF) Search in Prokaryotes. In prokaryotes, TFs (green) can move by 3D diffusion, sliding, hopping, and intersegmental transfer to reach their binding sites (red). These movements can be restricted by roadblocks due to the presence of architectural proteins (yellow) or other DNA-binding proteins (purple).

TFs can be split into two classes. Some TFs have general gene regulatory functions, control bacterial chromosome architecture, and typically bind with loose specificity. TFs of the second class regulate very limited sets of genes and often have stringent sequence specificity [2]. As a consequence, they typically have only very few specific binding sites in the whole genome; for example, the Lac repressor (LacR) has only three known binding sites in the *E. coli* genome and exclusively regulates the Lac operon [18].

The LacR has been used as a paradigmatic TF to investigate how TFs search the genome to find their sites. Gilbert and colleagues first isolated the LacR and described its DNA-binding properties [19]. Shortly thereafter, the LacR association rate to its specific binding site was reported to be much higher than would be expected if it would only diffuse in 3D [20]. In the early 1970s, several studies measured the equilibrium dissociation rate constants of the LacR on DNA with and without a Lac operator, demonstrating its ability to nonspecifically bind DNA [21–23]. Theoretical [12,24] and subsequent experimental [25,26] studies suggested that nonspecific DNA binding allows the LacR to find its main binding site faster than would be expected by diffusion alone, which gave rise to the concept of 'facilitated diffusion'. This model suggests that once bound nonspecifically, the LacR can perform sliding on DNA, hopping (local jumps on the same DNA segment), and intersegmental transfer (jump to a physically close piece of DNA but distant in 1D space) to increase its search efficiency (Figure 1).

Moving away from statistical ensemble measurements toward the observation of individual molecule behavior has allowed a leap forward in the understanding of TF–DNA interactions. By performing time-lapse single-molecule imaging of a fluorescently labeled LacR in live *E. coli*, Elf

and colleagues reported the first direct evidence for LacR DNA sliding resulting in facilitated diffusion *in vivo*. They found that LacR molecules spend ~90% of the time bound nonspecifically to DNA [27] and that it takes about 5 minutes for one molecule of LacR to find its binding site, which is too short for a purely 3D diffusion-mediated search. Elf's team later directly demonstrated that the LacR performs facilitated diffusion through 1D sliding over 45 bp, on average, *in vivo* [28]. They also found that the LacR often 'misses' its target when sliding, illustrating the trade-off between sliding speed and the ability to physically associate with a specific binding site. Elf's team also demonstrated that while sliding is essential for facilitated diffusion of the LacR, hopping and intersegmental transfer has a negligible impact [29]. However, this conclusion may not be generalizable, since TFs with higher nonspecific DNA affinity may benefit from intersegmental transfer to avoid being trapped for prolonged time periods on a small portion of the genome [29].

Searching the Eukaryotic Genome

In eukaryotes, the situation is far more complex both in *cis* (chromatin) and *trans* (TF specificity) (Figure 2). Eukaryotic genomes are typically several orders of magnitude larger than prokaryotic genomes and often contain hundreds of thousands of potential specific binding sites for each TF. A large fraction of the genome is wrapped around nucleosomes, which constitute an obstacle

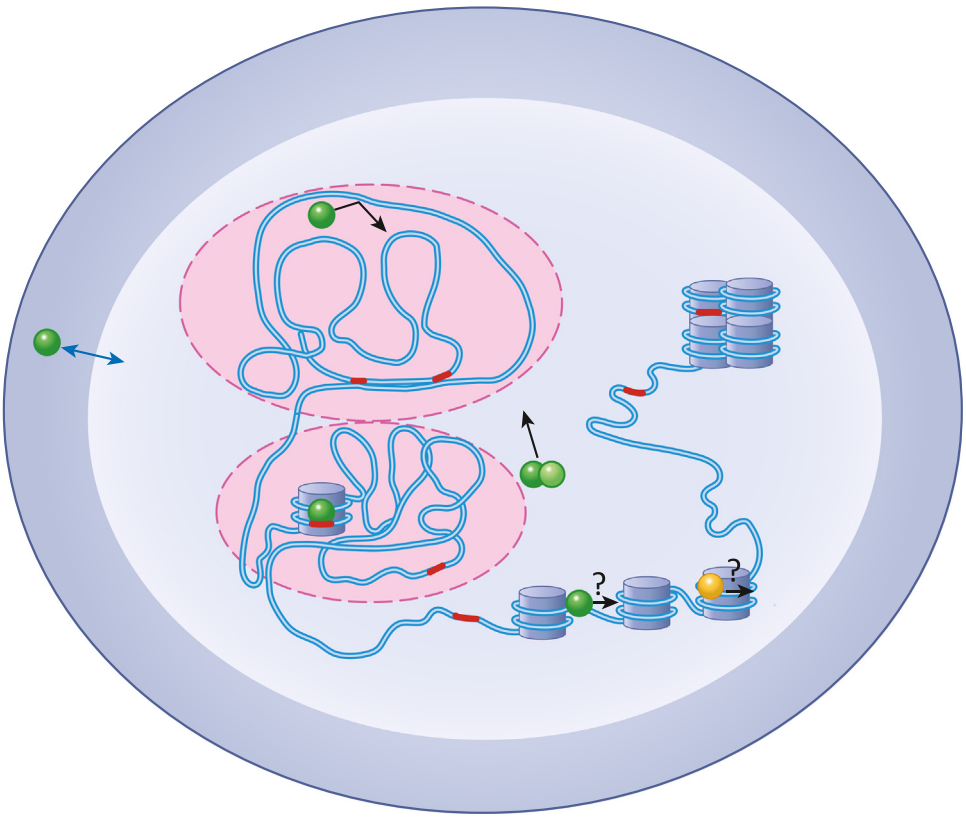

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Figure 2. Transcription Factor (TF) Search in Eukaryotes. In eukaryotes, there are a large number of binding sites (red) for each TF that can be located in different chromatin contexts. Some TFs (pioneer TFs; yellow) can interact with nucleosomal DNA. Whether eukaryotic TFs display any sliding between or on nucleosomes is unclear. The partitioning of the genome in topologically associating domains (pink halos) may partially confine TF molecules within subcompartments of the nucleus.

to TF–DNA interactions. Nucleosome density can vary considerably between sparsely occupied regions in active genes to very high nucleosome occupancy regions in heterochromatin [1]. The eukaryotic genome is partitioned into compartments of different sizes, allowing preferential physical interactions in *cis* within compartments [30]. Compared with gene-specific prokaryotic TFs, eukaryotic TFs have a rather loose specificity for their binding sites (i.e., each TF often binds to a large number of similar but not identical sequences with different affinities) [31]. This makes the definition of specific versus nonspecific sites blurrier and poses important challenges in identifying specific TF–DNA interactions.

Interactions with Nucleosomal DNA

Eukaryotic TFs differ strongly in their abilities to bind to specific DNA sites that are wrapped around nucleosomes. Those capable to do so are called 'pioneer TFs', and they can evict nucleosomes either directly or indirectly through the recruitment of chromatin remodelers [32]. Electromobility shift assay (EMSA) and more recently systematic evolution of ligands by exponential enrichment (SELEX) (Box 1) on nucleosomes [33] allow one to interrogate both specific and nonspecific interactions of TFs with nucleosomal DNA at equilibrium [34], and they have been used to compare pioneer properties of different TFs [33,35,36]. Progress in reconstituting chromatin *in vitro* with different degrees of compaction and histone modifications [37] now allows one to study the dynamics of chromatin exploration by pioneer TFs at the single-molecule level. This approach was used recently by Mivelaz and colleagues to dissect the interaction dynamics of the yeast pioneer transcription factor Rap1 with DNA wrapped into chromatin fibers [38]. They could discriminate transient nonspecific from specific DNA binding events on both nucleosomal and free DNA. They found that search efficiency of Rap1 on nucleosomal DNA was similar to naked DNA; however, its dwell time was

Box 1. Methods to Measure Transcription Factor (TF)–DNA interactions

1. *In vitro* approaches

- **E**lectro**M**obility **S**hift **A**ssay (EMSA): this approach is based on the retardation in the migration of a DNA fragment by its association to DNA-binding proteins. It allows measuring the K_d of specific and nonspecific DNA binding.
- **S**ystematic **E**volution of **L**igands by **EX**ponential enrichment (SELEX): this is the method of choice to identify the DNA-binding motif of a TF. The recent development of high-throughput SELEX [80] allows one to determine the binding preferences of hundreds of TFs on naked or nucleosomal DNA in parallel [33].
- **M**echanically **I**nduced **T**rapping **O**f **M**olecular **I**nteractions (MITOMI): MITOMI involves the trapping of TFs together with DNA sequences in very small volumes in a microfluid device, combined with a fluorescence readout of TF–DNA interactions [81].
- Single-molecule imaging by **T**otal **I**nternal **R**eflection **M**icroscopy (TIRF): TIRF microscopy is based on illuminating a very thin section at the surface of a sample, allowing very high signal-to-noise measurements. It is used for *in vitro* experiments to measure interactions of single molecules of TFs with DNA on the surface of a glass slide.

2. *In vivo* approaches

- ChIP-seq and **C**leavage **U**nder **T**argets and **R**elease **U**sing **N**uclease (CUT&RUN): ChIP-seq [82] and CUT&RUN [83] allow genome-wide mapping of TF–DNA interactions by isolating TFs bound to DNA fragments using an antibody targeted to the TF, following by amplification and high-throughput sequencing of the DNA fragments.
- **F**luorescence **R**ecovery **A**fter **P**hotobleaching (FRAP) [84]: this method involves the bleaching of a population of fluorescently labeled TFs in the nucleus followed by measuring how fast fluorescence recovers, which contains information about TF k_{off}, k_{on}, and diffusion rates.
- **F**luorescence **C**orrelation **S**pectroscopy (FCS) [85]: FCS involves measuring fluorescence intensity fluctuations of a subfemtoliter volume within live cells. Since this volume contains only a few fluorescent molecules at a given time point, it allows one to determine [*TF*] and diffusion rates.
- Single-molecule imaging/single-particle tracking (SPT) [27,42,43]: the recent development of electron multiplying charge-coupled device and scientific complementary metal–oxide–semiconductor cameras and illumination schemes allowing light sectioning of eukaryotic nuclei to decrease out of focus illumination and background signal allows one to visualize single molecules of TFs in live prokaryotic and eukaryotic cells. This has become the method of choice to simultaneously determine TF diffusion rates, fraction of DNA-bound TF molecules, and k_{off} of specific and nonspecific TF–DNA interactions.

shortened in the presence of a nucleosome. This suggests that k_{off} but not k_{on} of Rap1 is affected by chromatin state. By contrast, *in vivo* studies in yeast have suggested that the RSC (remodeling the structure of chromatin) chromatin remodeler can increase the k_{on} of the Ace1p transcription factor binding to its promoter and increases its k_{off}, which might be due to competition between the remodeling complex and TF binding [39]. In the case of the Oct4 and Sox2 TFs in pluripotent stem cells [40] as well as bicoid in *Drosophila* [41], chromatin accessibility was also suggested to directly impact their k_{on}. These discrepancies between *in vitro* and *in vivo* studies could be related to changes in chromatin accessibility upon TF binding *in vivo* that may alter DNA binding activity of competing TFs or chromatin remodelers. However, technical challenges in discriminating specific and nonspecific DNA binding events might also explain these differences. *In vitro*, the number, affinity, and position of specific sites relative to nucleosomes are known. By contrast, TFs can bind to a much broader diversity of specific sequences *in vivo*, which may be located at different positions with respect to nucleosomes that may differ in their post-translational modifications. As a consequence, k_{off} can vary considerably between specific sites [38], which makes it challenging to define unique criteria to identify specific DNA binding events *in vivo*.

Nonspecific DNA Binding

EMSAs have revealed large differences in nonspecific DNA binding properties between eukaryotic TFs [35,36]. Live-cell single-particle tracking (SPT) (Box 1) in eukaryotic cells [42–44] is used to discriminate between specific and nonspecific DNA binding events, based on their different dwell times (seconds to minutes for specific events and <1 s for nonspecific events [45]). However, and as mentioned above, it remains unclear to what extent this definition holds true in different chromatin contexts. The fraction of nonspecific DNA binding events not captured by any current approach is also unknown. Even though SPT can detect a subset of nonspecific DNA binding events, current limitations in camera speed and spatial resolution may prevent the identification of extremely short-lived TF–DNA interactions, and they do not allow one to discriminate binding events from slow free diffusion or local steric trapping.

Single-molecule imaging and EMSAs are also work-intensive and thus not easily amenable to determining nonspecific DNA binding properties of a large number of TFs. Recently, several studies have shown that TFs vary largely in their colocalization with mitotic chromosomes [46–52] and that this property is mainly driven by nonspecific DNA association [46,47,50,52]. Raccaud and colleagues quantified mitotic chromosome binding for 501 TFs, which they found to be predictive of the fraction of specific sites occupied by each TF at a given concentration. Surprisingly, TF-specific site occupancy was found to vary over three orders of magnitude for different TFs [52]. Differences in k_{off} and [*TF*] were small in comparison, and differences in [*BS*] could not explain these results. This suggests that distinct nonspecific DNA binding properties can result in large differences of k_{on} between TFs.

What are the mechanistic links between nonspecific DNA binding and TF search efficiency in eukaryotic cells? In principle, DNA sliding length and speed, local hopping, and intersegmental transfer could all contribute to TF search efficiency. There is currently no technology allowing one to discriminate these nanoscale dynamics *in vivo*. While we do have relatively detailed, quantitative data of LacR sliding dynamics, differences between TF–DNA interactions and the chromatin landscape between prokaryotes and eukaryotes make them poorly predictive of mammalian TF behavior. TFs such as p53 [53] and Sox2 [40] have the ability to slide on DNA *in vitro*; however, whether this also happens in the context of live mammalian cells is unknown. In contrast to LacR that spends 90% of its time associated to DNA, most eukaryotic TFs investigated so far spend >50% of their time freely diffusing in the nucleus [40,54–56], with some notable exceptions [57]. The contribution of hopping and intersegmental transfer might also be different from the

prokaryotic situation because of the specific 3D architecture of eukaryotic genomes [58]. Sequences flanking specific sites may also help or hinder the search process, depending on their TF affinity, and thereby significantly contribute to differences in the occupancy of different TF binding sites in the genome [59,60].

TF Compartmentalization

[*TF*] can vary over several orders of magnitude and is thereby a major tuning parameter for specific binding site occupancy. However, [*TF*] is often poorly characterized and can vary substantially between different regions in the nucleus. TFs either can be constitutively localized in the nucleus or can shuttle between the cytosol and the nucleus (Figure 3A). Some TFs are enriched in condensed chromatin regions, while others are more homogeneously filling the nucleus [52] (Figure 3B,C). At the nanoscale level, TFs have been shown to assemble local condensates in contact to cis-regulatory elements through interactions between their unstructured transactivation domains (Figure 3D). This not only increases their local concentration but also can decrease their k_{off} [61,62]. The formation of these structures is thought to be mediated by phase separation of unstructured domains of TFs, although a solid proof of how this mechanism operates *in vivo* is still pending [63]. Clustering of TFs is also suggested to increase the frequency

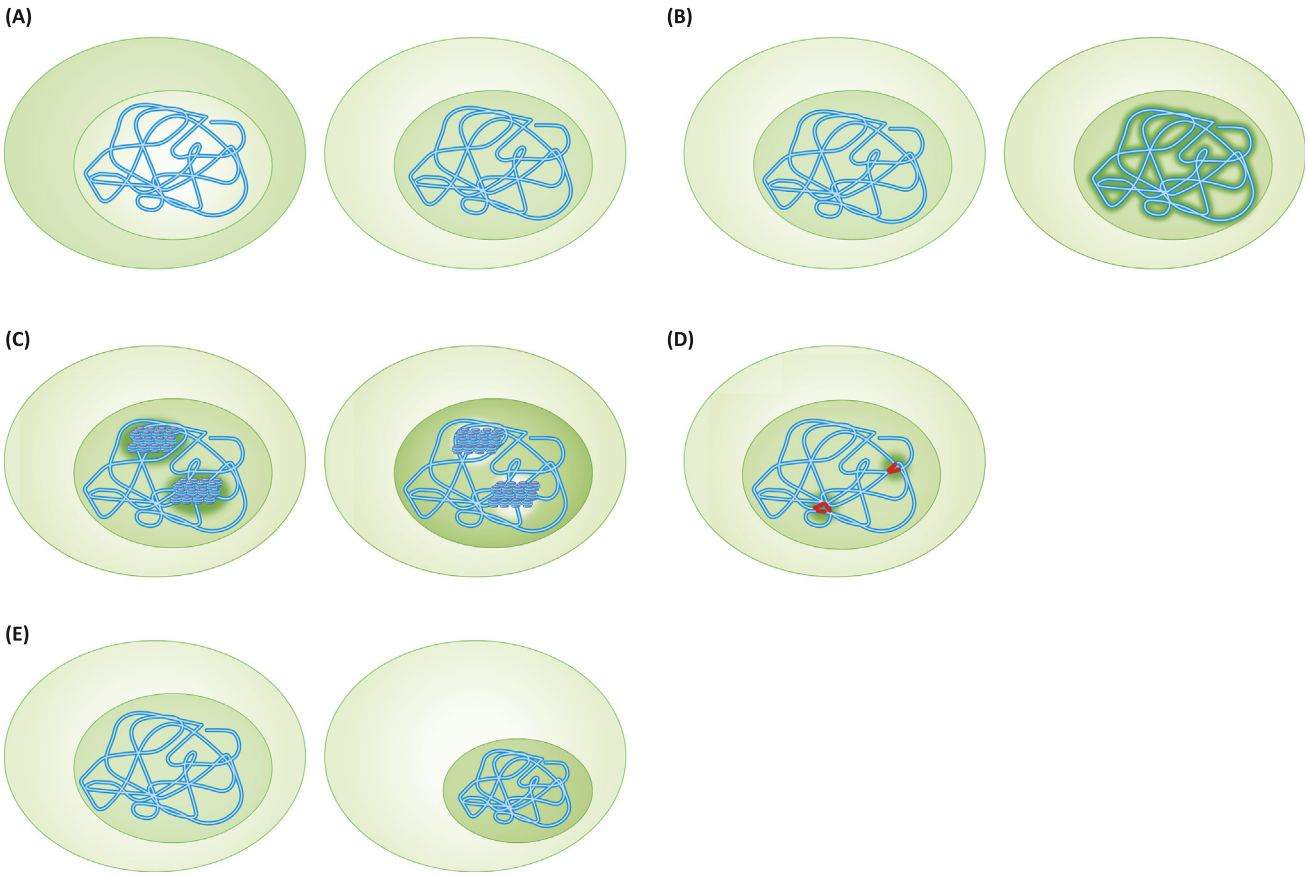

Figure 3. Compartmentalization of Transcription Factors (TFs) in the Nucleus. (A) TF concentration can change by modulating shuttling between the cytosol and nucleus. (B) TFs can vary in their colocalization with regions of different DNA densities. (C) Some TFs can display differential concentration in euchromatin versus heterochromatin regions. (D) TFs can form biomolecular condensates close to active regions rich in binding sites. (E) Changes in the size of the nucleus can alter both [*TF*] (concentration of TF) and [*BS*] (concentration of binding sites) and thus TF search efficiency.

of TF binding through bridging different parts of the genome and allowing intersegmental transfer of TFs [64]. Changing the volume in which TFs and DNA are contained can also modulate TF search efficiency (Figure 3E). The decreasing nuclear volume taking place during early zebrafish development was shown to increase the fraction of DNA-bound TFs by increasing both [*TF*] and [*BS*] [65]. The local genome architecture is also invoked as an important regulator of TF search efficiency. Since active and silent compartments are segregated in the nucleus, this suggests a role for TF trapping in active regions as supported by recent theoretical studies [58]. CTCF (CCCTC-binding factor) displays local trapping mediated by its RNA-binding domain, which allows one to preferentially scan some regions of the genome [66]. Remarkably, a CTCF mutant devoid of its RNA-binding domain displayed a decreased search efficiency but an unaltered k_{off}, demonstrating that association and dissociation rates can be modulated by distinct TF domains.

TF Cooperativity

TF cooperativity adds an additional layer of complexity in determining TF–DNA interaction dynamics. The formation of TF heterodimers generally increases their affinity for binding to composite DNA sites *in vitro* [1]. *In vivo*, the situation is often more complex because of indirect cooperativity through other DNA binding proteins or through modulation of chromatin accessibility. This can lead to TFs competing for the same binding site to paradoxically increase the binding of each other by 'assisted loading'. In this situation, the increase in local chromatin accessibility mediated by each competing TF overcomes the effect of direct competition to interact with the specific site [67].

We generally know a lot about how cooperativity increases the affinity of TFs for their binding sites by modulating their K_d. However, the respective impact of cooperativity on k_{off} and k_{on} is much less clear. Oct4 and Sox2 offer one interesting example of the complex relationship between cooperativity, k_{off}, and k_{on} in different contexts. These two TFs bind to thousands of composite motifs as a heterodimer in pluripotent stem cells and exhibit strong direct cooperativity *in vitro* [68]. Sox2 is efficient in searching for its binding sites when expressed outside of its natural context, in contrast to Oct4 [52]. Oct4 search efficiency can be substantially increased by coexpressing Sox2 [40,52]. By contrast, while the interaction of Sox2 with specific sites is stabilized by Oct4, its search efficiency seems to be mostly independent of Oct4 [40]. Chen *et al*. [40] have proposed that the Sox2-Oct4 heterodimer assembles in an ordered manner on its binding sites; however, the validity of these conclusions has been challenged by others [69]. In the context of *in vitro* reconstituted nucleosomes, Oct4 increases the propensity of Sox2 to bind to different positions of the nucleosomal surface [70]. Finally, when examined in their physiological context, the cooperativity exhibited by Oct4 and Sox2 stems mainly from local modulation of chromatin accessibility rather than direct interactions on DNA [71]. This illustrates how multiple layers of cooperativity can regulate TFs' search capacity, the stability of their specific DNA interactions, and their nucleosomal/naked DNA occupancy.

Concluding Remarks

The principles underlying TFs' search for their specific binding sites are now fairly well understood in the specific case of LacR in prokaryotes, but they are largely unclear in eukaryotes (see Outstanding Questions). A large number of parameters can in principle affect search efficiency, at the levels of both TF structure/biochemistry and chromatin organization. New imaging modalities allowing one to track TF motions on chromatin and in living cells have allowed important progress in describing the scales at which dynamic parameters operate. However, measurements of these parameters are work-intensive and suffer from limited temporal and spatial resolution. Determining k_{on} of TFs *in vivo* remains particularly delicate due to the limited information on TF concentrations, the number of binding sites, and the classification of specific versus

Outstanding Questions

How and to what extent are TF biochemical properties optimized for search efficiency?

How do eukaryotic TFs move on chromatin *in vivo*?

What is the role of 3D genome architecture in partitioning TFs in different regions of the genome?

What are the physical and chemical bases for nanoscale TF clustering?

Is there an evolutionary rationale for the vastly different nonspecific DNA binding properties and search capacities of different TFs?

nonspecific DNA-binding events in different chromatin contexts. The combination of *in vitro* measurements using highly controlled synthetic chromatin with *in vivo* experiments at high temporal and spatial resolution will be required to significantly advance the field. In that regard, new imaging modalities with improved spatial and/or temporal resolution for SPT, such as lattice light sheet microscopy [72], target-locking nanoscopy [73], or MINFLUX (minimal photon fluxes) [74], hold great promise. Further progress in determining TF concentrations will also be required, and recent methodological improvements show great promise in this regard [75–79]. This will hopefully bring us closer to understanding how TFs evolved different dynamic properties and coupled these to the control of their concentration and binding partners to regulate their activity.

Acknowledgments

I thank Christof Gebhardt and Beat Fierz for their critical reading of the manuscript and useful suggestions.

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