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Noncooperative Interactions between Transcription Factors and Clustered DNA Binding Sites Enable Graded Transcriptional Responses to Environmental Inputs

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SUMMARY

A paradigm in transcriptional regulation is that graded increases in transcription factor (TF) concentration are translated into on/off transcriptional responses by cooperative TF binding to adjacent sites. Digital transcriptional responses underlie the definition of anatomical boundaries during development. Here we show that NF-κB, a TF controlling inflammation and immunity, is conversely an analog transcriptional regulator that uses clustered binding sites noncooperatively. We observed that increasing concentrations of NF-κB are translated into gradual increments in gene transcription. We provide a thermodynamic interpretation of the experimental observations by combining quantitative measurements and a minimal physical model of an NF-κB-dependent promoter. We demonstrate that NF-κB binds independently to adjacent sites to promote additive RNA Pol II recruitment and graded transcriptional outputs. These findings reveal an alternative mode of operation of clustered TF binding sites, which might function in biological conditions where the transcriptional output is proportional to the strength of an environmental input.

INTRODUCTION

A commonly held view is that genes exist in only two alternative functional states, namely active or inactive, with enhancers controlling the probability but not the rate of transcription (Walters et al., 1995). In this view, a graded increase in transcription factor (TF) concentration is translated into a digital (on/off) transcriptional response, enabled by cooperative TF binding to adjacent sites (Segal et al., 2008) (Figure 1). This paradigm applies well to TFs operating in developmental processes, notably embryonic segmentation, which require the definition of sharp anatomical borders. A classic example is the morphogen model of Bicoid (Bcd) action in *Drosophila* embryogenesis. Cooperative binding of Bcd to adjacent DNA sites represents the molecular basis by which a gradual protein gradient of Bcd along the anterior-posterior embryonic axis is converted into a sharp on/off pattern of expression of Bcd target genes (Burz et al., 1998; Gregor et al., 2007). Nonetheless, clusters of adjacent binding sites for a given TF ("homotypic clusters"), as well as heterotypic clusters containing binding sites for multiple TFs, are a widespread genomic feature of higher eukaryotes (Arnone and Davidson, 1997; Berman et al., 2002; Lifanov et al., 2003; Markstein et al., 2002), and it is not intuitively obvious that on/off switches arising from the cooperative usage of such clustered sites may be compatible with biological responses other than those requiring the definition of well-demarcated gene expression domains.

Responses to environmental perturbations are conditions where the degree of gene activation is commensurate with the strength of the inducing stimulus. A well-known example is the inflammatory response induced by microbes, in which the intensity of the response is proportional to the microbial load. As another example, the p53 response to X or UV rays increases in proportion to the intensity of irradiation (Lahav et al., 2004). In principle this linear input/output (i/o) relationship may be achieved in at least three different ways: (1) by devising transcriptional regulatory circuits allowing transcription to be tuned to the intensity of the stimulus at the level of individual cells, (2) by increasing the fraction of cells in a population that are digitally activated in response to increasing doses of stimulus (Podtschaske et al., 2007), or (3) by increasing the number of consecutive pulses of gene activation occurring in individual cells in response to increasing doses of the stimulus (Lahav et al., 2004). The first of these models requires that the system be

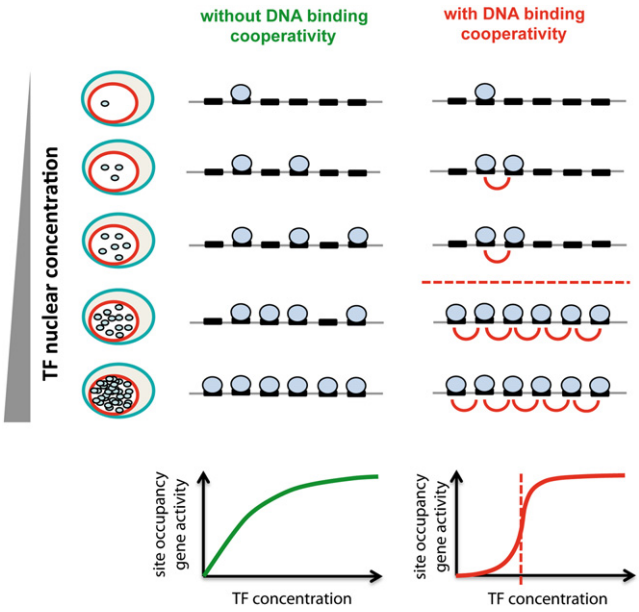

Figure 1. DNA Binding Cooperativity Generates Sharp Transcriptional Boundaries

If a TF binds to a cluster of DNA binding sites in a noncooperative manner, a gradual increase in TF concentration generates a gradual increase in the average occupancy of the cluster. Conversely, if TF binding to adjacent sites is cooperative, a gradual increase in TF concentration generates a "digital" on/off response as the concentration sweeps a threshold value (dashed line). The higher binding cooperativity, the steeper the transition between the "off" and the "on" states.

able to accurately sense, transduce, and exploit at the transcriptional level the regulatory information provided by a broad range of concentrations of the stimulus. This highly intuitive and rational operational mode, for which no unequivocal evidence has been provided, implies a continuous spectrum of outputs in response to a continuous spectrum of inputs. Cellular responses to cytokines, whose concentration in body fluids varies across orders of magnitude in response to stimulation, should provide an ideal experimental setting to challenge this model.

NF-κB/Rel is a family of dimeric TFs conserved from flies to humans that controls transcription of hundreds of genes implicated in inflammatory and immune responses (Hayden and Ghosh, 2008). The most common NF-κB dimer in mammalian cells is p50/p65, which binds to decameric DNA sequences (Natoli et al., 2005). p50/p65 and most other NF-κB dimers are sequestered in the cytoplasm of unstimulated cells by association with inhibitory proteins, the IκBs. In order to allow nuclear translocation of NF-κB in response to stimulation, the IκBs are phosphorylated and degraded. IκBα (encoded by the *NFKBIA* gene) is both the most rapidly degraded IκB protein and the most rapidly resynthesized one, by virtue of a negative feedback based on NF-κB binding and activation of the *NFKBIA* promoter (Hoffmann et al., 2002).

The *D. melanogaster* Rel family TF Dorsal cooperates with itself and other TFs (like Twist) to establish sharp patterns of expression of its target genes during embryo development (Ip et al., 1992; Stathopoulos and Levine, 2002), indicating that selected NF-κB/Rel TFs can direct digital patterns of developmental gene expression using cooperative interactions (Gonzalez-Crespo and Levine, 1993; Roth et al., 1989). Importantly, when Dorsal-deficient embryos were reconstituted with Dif, a Rel family protein involved in the immune response, Dorsal target genes were activated, but the spatial precision arising from cooperative binding of Dorsal to DNA was lost (Stein et al., 1998). Thus, even closely related TFs binding to the same or similar DNA motifs but operating in different biological contexts exhibit different properties regarding cooperativity in binding.

Here we report that, in contrast with the classical models of on/off developmental gene regulation, clusters of NF-κB binding sites are organized to generate transcriptional outputs whose intensity varies with that of the stimulus. The interpretation of the response curves in terms of a thermodynamic model adapted to the analysis of TF-DNA interactions indicates that the graded response is a consequence of noncooperative binding of NF-κB to clustered sites in *cis*-regulatory elements (CREs). We propose that such an analog type of regulation may best fit the requirements of most TFs controlling responses to environmental stimuli.

RESULTS

Genome-wide Analysis of Clusters of Conserved NF-κB Binding Sites

We began by investigating the presence of evolutionarily conserved clusters of NF-κB binding sites ("κB sites") in the proximal promoter regions (−1000 bp to +200 bp relative to transcription start sites, TSSs) of 24,398 human RefSeq genes. κB sites were predicted using an available NF-κB position weight matrix (PWM) (Grilli et al., 1993). Phylogenetic conservation has been shown to provide a useful filter to enrich for functional binding sites identified in genome scans (Gumucio, 1991), although a sizeable fraction of active regulatory elements lacks detectable sequence constraints (Birney et al., 2007; Ponting, 2008). In our initial genome scan we used a simple, relatively strict phylogenetic filter, requiring that for each κB site identified in the human genome a κB site was also found in the aligned sequence of more than half of the 11 mammalian genomes examined.

We identified 24 clusters comprising three or more conserved κB sites upstream of 23 genes (see Table 1). Comparing this list of genes to a curated list of NF-κB targets (from http://nfkb.org/) we found that it was significantly enriched for known NF-κB target genes ($p = 1.6 \times 10^{-10}$, Fisher's exact test). Of note, this short list contained two known negative regulators of NF-κB signaling: *NFKBIA*, encoding IκBα, was one of the two top-scoring genes in the genome with five deeply conserved κB sites (see Figure 2A); and *TNFAIP3*, encoding the negative regulator A20 (Werner et al., 2008), had three κB sites in its promoter. Also identified were two members of the Rel family itself: *REL*, encoding the c-Rel protein, had three κB sites; and two different isoforms of *NFKB2*, encoding p105/p50, had clusters of three κB sites.