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Regulation of Gene Expression via the Core Promoter and the Basal Transcriptional Machinery

Tamar Juven-Gershon[a,b] and **James T. Kadonaga**[a,*]

[a] Section of Molecular Biology, 0347, University of California, San Diego, La Jolla, CA 92093-0347, USA

Abstract

The RNA polymerase II core promoter is a structurally and functionally diverse transcriptional regulatory element. There are two main strategies for transcription initiation – focused and dispersed initiation. In focused initiation, transcription starts from a single nucleotide or within a cluster of several nucleotides, whereas in dispersed initiation, there are several weak transcription start sites over a broad region of about 50 to 100 nucleotides. Focused initiation is the predominant means of transcription in simpler organisms, whereas dispersed initiation is observed in approximately two thirds of vertebrate genes. Regulated genes tend to have focused promoters, and constitutive genes typically have dispersed promoters. Hence, in vertebrates, focused promoters are used in a small but biologically important fraction of genes. The properties of focused core promoters are dependent upon the presence or absence of sequence motifs such as the TATA box and DPE. For example, Caudal, a key regulator of the homeotic gene network, preferentially activates transcription from DPE- versus TATA-dependent promoters. The basal transcription factors, which act in conjunction with the core promoter, are another important component in the regulation of gene expression. For instance, upon differentiation of myoblasts to myotubes, the cells undergo a switch from a TFIID-based transcription system to a TRF3-TAF3-based system. These findings suggest that the core promoter and basal transcription factors are important yet mostly unexplored components in the regulation of gene expression.

Introduction

The core promoter lies at the center of the transcription process, yet it is often an overlooked component in the regulation of gene expression (for reviews, see: Smale and Kadonaga, 2003; Thomas and Chiang, 2006; Heintzman and Ren, 2007; Juven-Gershon et al., 2008b). The core promoter is generally defined to be the DNA region that directs the accurate initiation of transcription by RNA polymerase II. In the past, the core promoter has often been presumed to be a generic entity that functions by a single universal mechanism, but it is now clearly apparent that there is widespread diversity in core promoter structure and function. In this review, we will discuss some key features of the RNA polymerase II core promoter, and provide some examples of the role of the core promoter and the basal transcription machinery in the regulation of gene expression.

Focused versus Dispersed Transcription Initiation

Examination of the patterns of transcription initiation reveals two different modes of transcription initiation – focused and dispersed (Fig. 1) (see, for example: Smale and Kadonaga,

*Corresponding author. Fax: +1 858 534 0555. jkadonaga@ucsd.edu (J.T. Kadonaga)..
[b]Current address: The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

2003;Carninci et al., 2006;Juven-Gershon et al., 2006a,2008b). In focused initiation, transcription starts at a single nucleotide or within a narrow region of several nucleotides, whereas in dispersed initiation, there are multiple weak start sites over a broad region of about 50 to 100 nucleotides. Some promoters exhibit the combined qualities of both focused and dispersed promoters – for instance, a promoter might have multiple dispersed start sites with one particularly strong start site. In this essay, however, we will limit the discussion to focused and dispersed promoters rather than the mixed mode promoters.

Focused transcription initiation occurs in all organisms, and appears to be the predominant or exclusive mode of transcription in simpler organisms. In vertebrates, however, about 70% of genes have dispersed promoters, which are typically found in CpG islands. It generally appears that focused promoters are associated with regulated genes, whereas dispersed promoters are used in constitutive genes. From a teleological standpoint, this arrangement is consistent with the notion that it would be easier to regulate the transcription of a gene with a single transcription start site than one with multiple start sites. Conversely, variations in the expression of a constitutive gene would be minimized by the use of multiple start sites.

Most mechanistic studies of RNA polymerase II transcription have been carried out with focused promoters. Although focused promoters comprise a minority of all promoters in vertebrates, there is an inordinate amount of effort devoted to focused promoters relative to dispersed promoters because of the biological significance of the regulated genes with which the focused promoters are associated. The analysis of focused core promoters has led to the discovery of sequence motifs such as the TATA box, BREu (upstream TFIIB Recognition Element), Inr (Initiator), MTE (Motif Ten Element), DPE (Downstream Promoter Element), DCE (Downstream Core element), and XCPE1 (X Core Promoter Element 1) (Fig. 2). In contrast, dispersed promoters generally lack BRE, TATA, DPE, and MTE motifs (Sandelin et al., 2007;Carninci et al., 2006). It is likely that there are fundamental differences in the mechanisms of transcription from focused versus dispersed promoters. For the remainder of this review, we will mainly describe studies of focused core promoters.

Basal Transcription Factors

The focused core promoter, which typically encompasses −40 to +40 relative to the +1 transcription start site, is the location at which the RNA polymerase II machinery initiates transcription. Purified RNA polymerase II can synthesize RNA from a DNA template, but is not able to recognize the core promoter. This process requires additional factors that are commonly known as the “general” or “basal” transcription factors, which include TFIIA (Transcription Factor for RNA polymerase II A), TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. These factors do not act in a “general” manner at all core promoters, and hence, we will refer to them as the “basal” transcription factors.

With TATA-driven core promoters, transcription can be achieved in vitro with purified RNA polymerase II, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. However, these same factors are not able to mediate transcription from a DPE-driven promoter (Lewis et al., 2005). In addition, NC2 (Negative Cofactor 2; also known as Dr1-Drap1), which was identified as repressor of TATA-dependent transcription, was found to be an activator of DPE-dependent transcription (Willy et al., 2000; Hsu et al., 2008). TATA- versus DPE-dependent transcription appears to be controlled, at least in part, by a simple circuit in which TBP (TATA box-Binding Protein) activates TATA transcription and represses DPE transcription, whereas NC2 and Mot1 (an ATPase that removes TBP from DNA) block TBP function (Hsu et al., 2008; van Werven et al., 2008) and thus promote DPE transcription and repress TATA transcription (Hsu et al., 2008).

TFIID is a key basal transcription factor that is involved in the recognition of focused core promoters (for review, see Thomas and Chiang, 2006). TFIID is a multisubunit complex that comprises TBP and about a dozen TAFs (TBP-Associated Factors). There are multiple potential points of interaction of TFIID with the core promoter. The TBP subunit binds to the TATA box, the TAF1 and TAF2 subunits recognize the Inr, the TAF1 subunit is in close proximity to the DCE, and the TAF6 and TAF9 subunits appear to interact with the DPE.

There are also mechanisms of core promoter recognition that do not involve the canonical TFIID complex. For instance, as discussed below, there are TRFs (TBP-Related Factors) with functions that are distinct from those of TBP.

The other known basal transcription factors participate in the early steps in transcription as follows. TFIIB interacts with TBP and assists in the recruitment of polymerase to the core promoter. TFIIB can bind to core promoter sequences at the BREu and BREd motifs in a manner that is dependent upon the binding of TBP to the TATA box (for review, see Deng and Roberts, 2007). TFIIA appears to promote the binding of TBP to the TATA box. TFIIE, TFIIF, and TFIIH act subsequent to the binding of TFIID and TFIIB to the core promoter, and mediate the unwinding of DNA and the early steps in the transcription process.

Core Promoter Motifs

The focused core promoter is diverse in terms of its structure and function. There are several known sequence motifs that can contribute to core promoter activity (Fig. 2), and it is likely that many other core promoter elements remain to be discovered. There are no universal core promoter elements. A brief overview of several core promoter motifs is as follows.

The Initiator (Inr)

The Inr encompasses the transcription start site, and is probably the most commonly occurring core promoter motif (Ohler et al., 2002; FitzGerald et al., 2006; Gershenzon et al., 2006). The function of the Inr as a distinct core promoter element was articulated by Smale and Baltimore (1989). Although several factors have been found to interact with the Inr, the binding of TFIID correlates best with Inr activity (discussed in Smale and Kadonaga, 2003). Functional analyses have determined that the Inr consensus is YYANWYY (IUPAC nucleotide code) in humans and TCAKTY in *Drosophila*. Inriceand *Arabidopsis*, a YR Inr motif (with R+1) has been identified (Yamamoto et al., 2007b). Inr-like sequences have also been described in *Saccharomyces cerevisiae* (Yang et al., 2007).

Computational analyses of *Drosophila* promoters have suggested an Inr consensus of TCAGTY (Ohler et al., 2002; FitzGerald et al., 2006), which is nearly identical to the *Drosophila* Inr consensus of TCAKTY determined via functional studies, such as the binding of TFIID (Purnell et al., 1994; Chalkley and Verrijzer, 1999). In contrast, computational analyses of mammalian promoters (both focused and dispersed) have led to a broader mammalian Inr consensus of YR (where R is +1; Carninci et al., 2006; Frith et al., 2008), which differs from the functional mammalian Inr consensus (YYANWYY). This difference is probably due to the high frequency (perhaps around 70%; Carninci et al., 2006; Kim et al., 2005; Cooper et al., 2006) of dispersed promoters in mammals and the inclusion of both dispersed and focused promoters in the computational analyses. The Inr consensus for focused mammalian promoters may more closely resemble the mammalian functional Inr consensus (YYANWYY) or even perhaps the more restrictive *Drosophila* Inr consensus (TCAKTY).

Focused transcription typically initiates within the Inr, and the A nucleotide in the Inr consensus is usually designed as the “+1” position, whether or not transcription actually initiates at that particular nucleotide. This convention is useful because other core promoter motifs, such as

the MTE and DPE, function with the Inr in a manner that exhibits a strict spacing dependence with the Inr consensus sequence (and hence, the A+1 nucleotide) rather than the actual transcription start site (Burke and Kadonaga, 1997; Kutach and Kadonaga, 2000; Lim et al., 2004).

The TATA Box and BRE Motifs

The TATA box is the first core promoter motif that was discovered (Goldberg, 1979) as well as the best known core promoter element. The metazoan TATA box consensus is TATAWAAR, where the upstream T is usually located at −31 or −30 relative to the A+1 (or G+1) position in the Inr (Carninci et al., 2006; Ponjavic et al., 2006). As noted above, the TATA box is recognized and bound by the TBP subunit of the TFIID complex. Both the TATA box and TBP are conserved from archaebacteria to humans (Reeve, 2003). The TATA box is also present in plants (Molina and Grotewold, 2005; Yamamoto et al., 2007a, 2007b). Although the TATA box is a well known core promoter motif, it is present in only about 10-15% of mammalian core promoters (Carninci et al., 2006; Kim et al., 2005; Cooper et al., 2006).

The BRE (TFIIB Recognition Element) was initially identified as a TFIIB binding sequence that is immediately upstream of a subset (~10-30%) of TATA box elements (Lagrange et al., 1998). In addition, a second TFIIB recognition site, the BREd (downstream TFIIB recognition element), was found immediately downstream of the TATA box (Deng and Roberts, 2005). The discovery of the BREd led to the renaming of the original BRE as BREu, for upstream BRE (reviewed in Deng and Roberts, 2007). Both the BREu and BREd function in conjunction with a TATA box, and have been found to increase as well as to decrease the levels of basal transcription (Lagrange et al., 1998; Evans et al., 2001; Deng and Roberts, 2005). More recent studies suggest a distinct role for the BREu in transcriptional regulation (Juven-Gershon et al., 2008a; discussed below).

DPE and MTE Motifs

The DPE (downstream core promoter element) was identified as a TFIID recognition site that is downstream of the Inr (Burke and Kadonaga, 1996). The DPE is located precisely from +28 to +33 relative to the A+1 and is conserved from *Drosophila* to humans (Burke and Kadonaga, 1997). The DPE does not appear to be present in *Saccharomyces cerevisiae*. The DPE is a recognition site for TFIID, which binds cooperatively to the Inr and DPE motifs. The spacing between the Inr and DPE is critical for transcriptional activity of DPE-dependent promoters (Kutach and Kadonaga, 2000). DPE-dependent promoters typically contain only DPE and Inr motifs. In some cases, however, TATA, Inr, and DPE motifs can be found in the same core promoter.

The MTE (motif ten element) was found to be a functionally active core promoter element that corresponds to an overrepresented sequence (termed motif 10) that was identified in *Drosophila* core promoter regions (Ohler et al., 2002; Lim et al., 2004). The MTE is located immediately upstream of the DPE at precisely +18 to +27 relative to the A+1 in the Inr, and is conserved from *Drosophila* to humans. DNase I footprinting analyses suggest that the MTE, like the DPE, is a recognition site for TFIID. The MTE functions cooperatively with the Inr, but can act independently of the DPE as well as the TATA box. There is, however, synergy between the MTE and DPE as well as between the MTE and TATA box.

These studies led to the design of a super core promoter (SCP) that contains a TATA, Inr, MTE, and DPE in a single promoter (Juven-Gershon et al., 2006b). The SCP is the strongest core promoter observed in vitro and in cultured cells, and yields high levels of transcription in conjunction with transcriptional enhancers. These findings indicate that gene expression levels can be modulated via the core promoter.

Role of the Core Promoter in the Regulation of Gene Expression

Transcriptional regulation is achieved not only by diversity in enhancers, but also by diversity in core promoter structure (see, for example: Smale, 2001; Butler and Kadonaga, 2002). This effect is seen, in particular, in the area of enhancer-promoter communication. For instance, the *Drosophila* AE1 and IAB5 enhancers preferentially activate the TATA-containing *even-skipped* core promoter relative to the TATA-less and DPE-containing *white* core promoter (Ohtsuki et al., 1998). In addition, DPE- as well as TATA-specific enhancers were identified in an enhancer-trapping screen in *Drosophila* (Butler and Kadonaga, 2001). Thus, some activators prefer TATA-dependent promoters, whereas others prefer DPE-dependent promoters.

More recently, the analysis of the *Drosophila* homeotic (Hox) genes has revealed new insights into the role of the core promoter in a regulatory network (Juven-Gershon et al., 2008a). In this study, it was found that nearly all of the Hox genes, which were previously known to have TATA-less promoters, contain DPE-dependent core promoters (Fig. 3). This observation suggested that at least some of the transcription factors that regulate the Hox gene network might be DPE-specific activators. Following this hypothesis, it was found that Caudal, a sequence-specific DNA-binding transcription factor and key regulator of the Hox genes, is a DPE-specific activator. In addition, Caudal-mediated activation of the *Antennapedia* P2 enhancer-promoter region as well as the *Sex combs reduced* enhancer-promoter region was observed to be dependent upon the DPE motifs in their respective core promoters. These findings collectively indicate an important role of the DPE in the regulation of the Hox genes.

Further investigation showed that the function of Caudal is more complex than a simple matter of specificity for the DPE relative to the TATA box. Specifically, the BREu motif suppresses the ability of Caudal to activate transcription via the TATA box but not the DPE. Hence, as depicted in Fig. 4, there are three levels of Caudal activation – strong activation via the DPE (in the presence or absence of a BREu), weaker activation via the TATA box in the absence of a BREu, and little or no activation via the TATA box with a BREu.

Hence, these studies of Caudal and the Hox genes reveal how specific core promoter motifs can play a central role in an important biological network. Yet, it is important to consider why Caudal might act as a DPE-specific activator. In a simple sense, it could be imagined that DPE specificity would be useful in the construction of regulatory networks. As in the wiring of a printed circuit board, there could be connections between transcriptional enhancers and their cognate core promoters. The use of DPE- and TATA-specific activators would enable the construction of more sophisticated and effective connections between enhancers and promoters (Fig. 5).

TBP-related Factors (TRFs) and Transcriptional Regulation

There is diversity not only in core promoter elements, but also in the basal transcription machinery. This concept is nicely exemplified in studies of the TBP-related factors (TRFs) (for reviews, see: Jones, 2007; Müller et al., 2007; Reina and Hernandez, 2007; Torres-Padilla and Tora, 2007). There are three TRFs, which are generally termed TRF1, TRF2, and TRF3.

TRF1 does not exist in yeast and humans, but is present in *Drosophila*. In many eukaryotes, including yeast and humans, TBP participates in transcription by RNA polymerases I, II, and III. However, in *Drosophila*, TRF1 is used instead of TBP for RNA polymerase III transcription (Takada et al., 2000).

TRF2 (also known as TLF, TLP, TRF, and TRP) is present in most eukaryotes, and is involved in transcription by RNA polymerase II. TRF2 does not bind to TATA box sequences, and

cannot replace TBP in vitro. It appears that many genes are regulated by TRF2 instead of TBP – one such example is the *Drosophila* histone H1 gene (Isogai et al., 2007). The TATA-less H1 linker histone gene is in a cluster of genes that also includes the four TATA-containing core histone genes, which are transcribed with TBP. These findings suggest the use of different transcriptional mechanisms within a cluster of genes.

TRF3 (also known as TBP2 and TBPL2) appears to be present only in vertebrates, and is the TRF that is most closely related to TBP. TRF3 can bind to TATA boxes and support TATA-dependent transcription (Bártfai et al., 2004; Jallow et al., 2004). TRF3 was found to be important for embryonic development (Bártfai et al., 2004; Jallow et al., 2004). In addition, zebrafish embryos that are depleted of TRF3 exhibit multiple developmental defects and fail to undergo hematopoiesis (Hart et al., 2007).

A particularly striking function of TRF3 was discovered during the analysis of the differentiation of myoblasts to myotubes (Deato and Tjian, 2007; Deato et al., 2008). Myoblasts were found to contain the canonical TBP-containing TFIID complex; however, upon terminal differentiation into myotubes, the TFIID complex was replaced by a TRF3-TAF3-containing complex (Fig. 6). These findings suggest that terminally differentiated cells may employ specialized transcription systems that are dedicated to the particular functions of the cells. It will be interesting to see if an analogous effect is observed in the differentiation of other cell types.

Conclusions and Perspectives

The core promoter and the basal transcriptional machinery are two important yet relatively unexplored dimensions in the regulation of gene expression. It is now apparent that diversity in the structure and function of core promoters and basal transcription factors contributes to developmental processes that lead to organismal complexity (Levine and Tjian, 2003). Thus, in the future, it will be essential to consider and to incorporate these factors in the analysis of gene regulation. For instance, transcriptional enhancers would ideally be studied in conjunction with their cognate core promoters. Alternatively, a new generation of reporter vectors could be designed with core promoters that function with both TATA- and DPE-specific enhancers (see, for example, Pfeiffer et al., 2008). The increased appreciation and understanding of core promoter motifs and basal transcription factors will lead to new and exciting discoveries, and ultimately, provide a more complete and accurate view of biological regulation.

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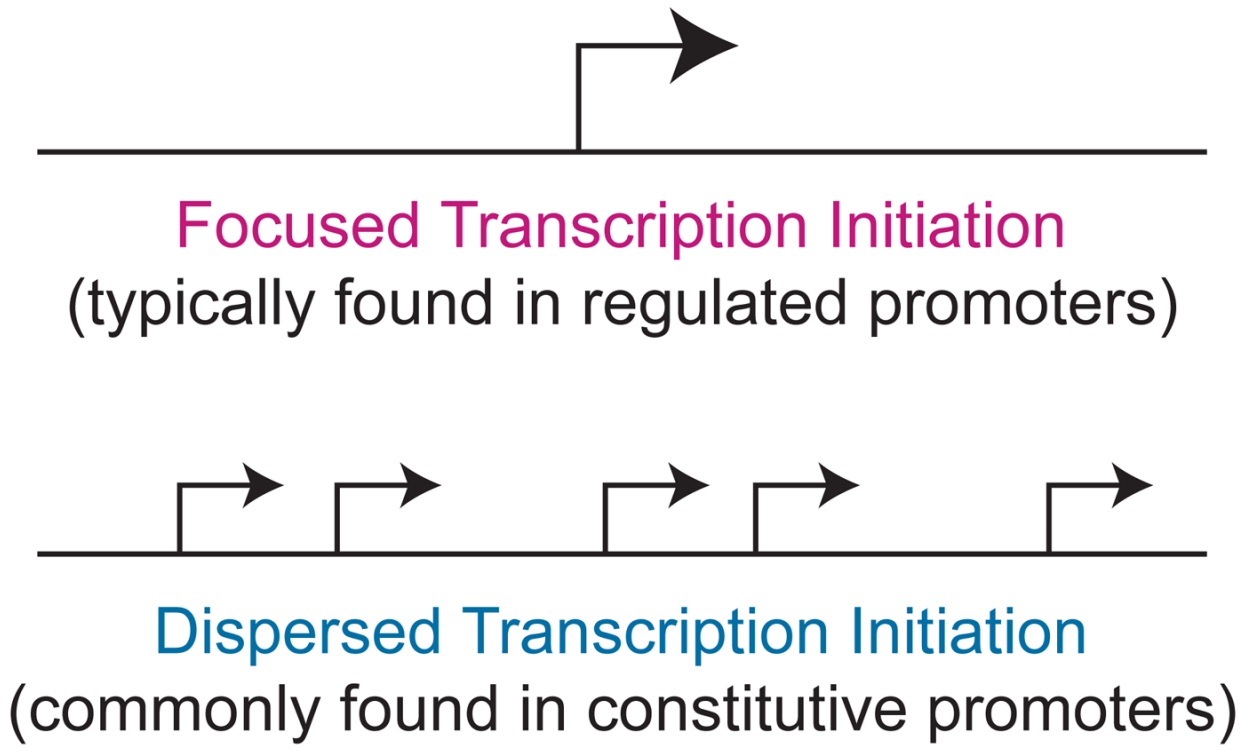

Fig. 1.

Focused versus dispersed transcription initiation. In focused transcription, there is either a single major transcription start site or several start sites within a narrow region of several nucleotides. Focused transcription is the predominant mode of transcription in simpler organisms. In dispersed transcription, there are several weak transcription start sites over a broad region of about 50 to 100 nucleotides. Dispersed transcription is the most common mode of transcription in vertebrates. For instance, dispersed transcription is observed in about two-thirds of human genes. In vertebrates, focused transcription tends to be associated with regulated promoters, whereas dispersed transcription is typically observed in constitutive promoters in CpG islands.

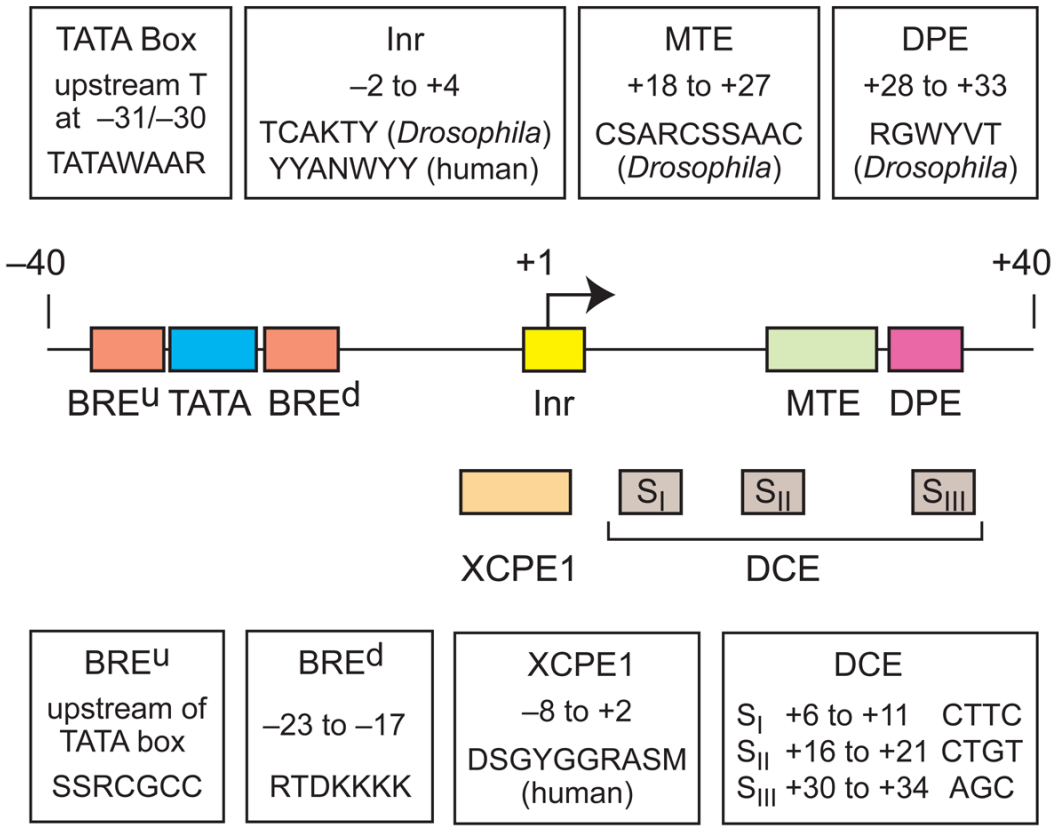

Fig. 2.

Some core promoter motifs for transcription by RNA polymerase II. This diagram is roughly to scale. These motifs are typically found in focused core promoters. There are no universal core promoter elements. It is likely that additional core promoter motifs remain to be discovered. The properties of any particular core promoter are dictated by the presence or absence of specific core promoter elements. For instance, as discussed in the text, a TATA-dependent core promoter with TATA + Inr motifs has different properties than a DPE-dependent core promoter with Inr + DPE motifs.

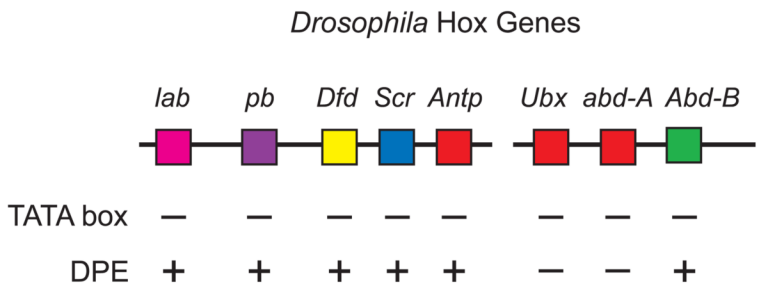

Fig. 3.

Nearly all of the *Drosophila* Hox genes contain TATA-less, DPE-dependent core promoters. The two most evolutionarily recent Hox genes, *Ubx* and *abd-A*, lack both TATA and DPE motifs. Both the upstream (P1) and downstream (P2) promoters of the *Antp* gene contain TATA-less, DPE-dependent core promoters. In the indicated DPE-dependent core promoters, the Inr and DPE sequences as well as the Inr-to-DPE spacing are conserved from *Drosophila melanogaster* to *Drosophila virilis*, which are separated by an evolutionary period of about 40 to 60 million years. *lab*, *labial*; *pb*, *proboscipedia*; *Dfd*, *Deformed*; *Scr*, *Sex combs reduced*; *Antp*, *Antennapedia*; *Ubx*, *Ultrabithorax*; *abd-A*, *abdominal-A*; Abd-*B*, *Abdominal-B*. Results taken from Juven-Gershon et al. (2008a).

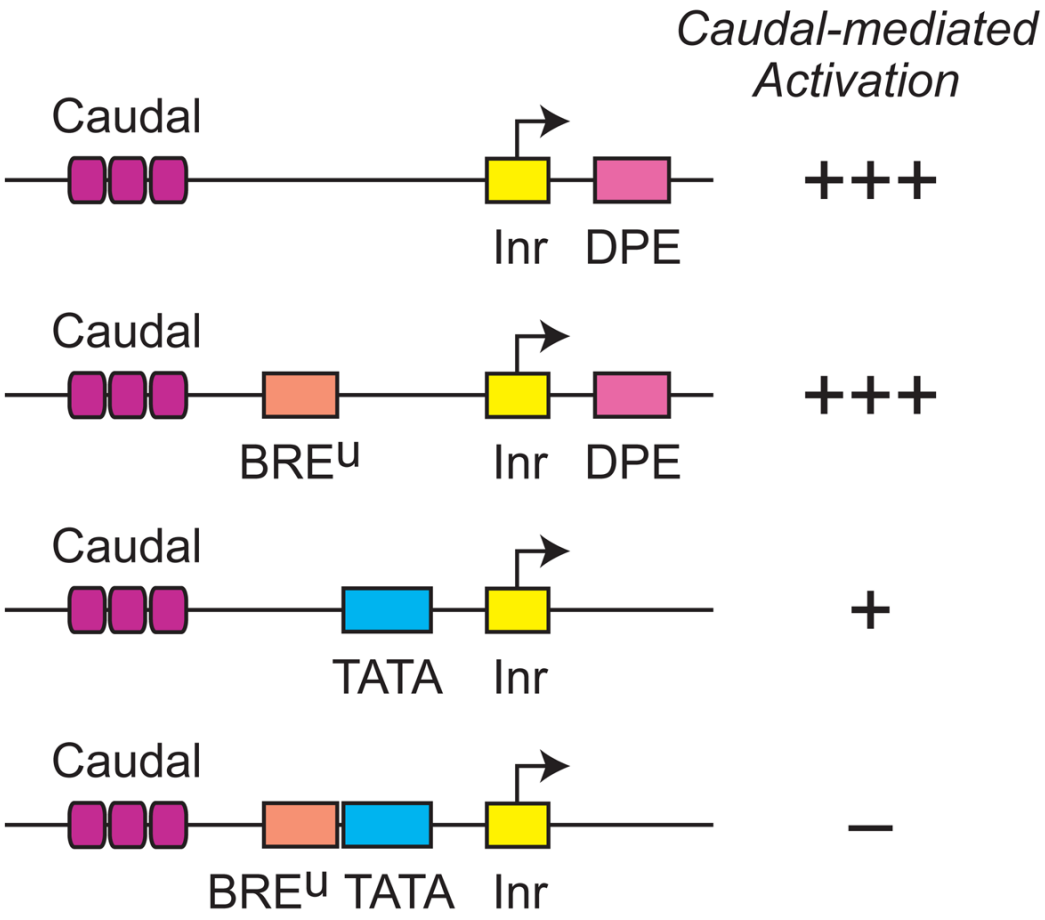

Fig. 4.

Caudal is a DPE-specific activator. Caudal preferentially activates transcription from DPE-dependent core promoters relative to TATA-dependent core promoters. In addition, the presence of a BREu motif upstream of the TATA box further suppresses the ability of Caudal to activate transcription. The BREu motif does not affect the ability of Caudal to activate a DPE-dependent core promoter. The TATA box also does not alter the ability of Caudal to activate transcription via the DPE motif (not shown). Results taken from Juven-Gershon et al. (2008a).

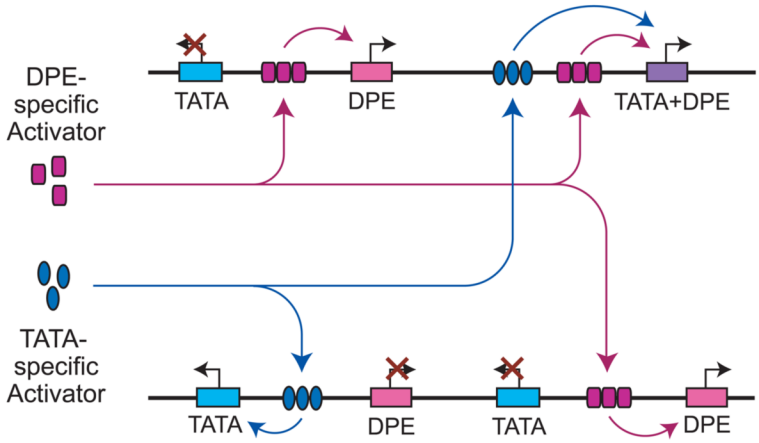

Fig. 5.

A simplified, hypothetical diagram of activation by DPE- and TATA-specific factors. Transcription factors bind to enhancers, but only activate transcription from promoters with the appropriate core promoter elements. The core promoter containing both TATA and DPE motifs can be activated by either DPE- or TATA-specific activators. Transcription levels can be further regulated by the presence of the BREu as well as other core promoter motifs.

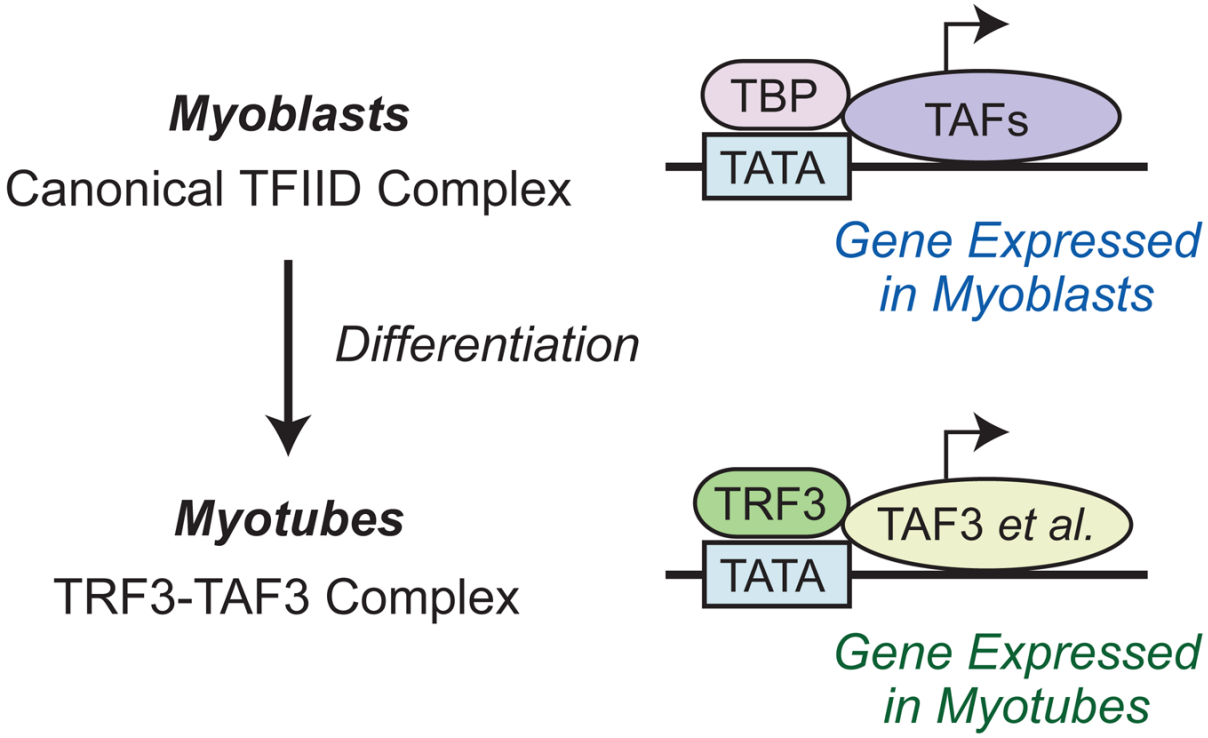

Fig. 6.

Replacement of the canonical TFIID complex by a TRF3-TAF3-containing complex upon terminal differentiation of myoblasts into myotubes. Both complexes bind to TATA box motifs via the TBP or TRF3 subunits. These findings exemplify the establishment of a new basal transcription system upon cell differentiation, and suggest that analogous processes may occur in other cell types. Results taken from Deato and Tjian (2007).

Mammalian RNA polymerase II core promoters: insights from genome-wide studies

Albin Sandelin[*‡], *Piero Carninci*[‡§], *Boris Lenhard*[‖], *Jasmina Ponjavic*[¶], *Yoshihide Hayashizaki*[‡§] *and David A. Hume*[#]

Abstract | The identification and characterization of mammalian core promoters and transcription start sites is a prerequisite to understanding how RNA polymerase II transcription is controlled. New experimental technologies have enabled genome-wide discovery and characterization of core promoters, revealing that most mammalian genes do not conform to the simple model in which a TATA box directs transcription from a single defined nucleotide position. In fact, most genes have multiple promoters, within which there are multiple start sites, and alternative promoter usage generates diversity and complexity in the mammalian transcriptome and proteome. Promoters can be described by their start site usage distribution, which is coupled to the occurrence of *cis*-regulatory elements, gene function and evolutionary constraints. A comprehensive survey of mammalian promoters is a major step towards describing and understanding transcriptional control networks.

Transcription start site
A nucleotide in the genome that is the first to be transcribed into a particular RNA.

Every aspect of homeostasis, growth, differentiation and development in eukaryotes requires the regulated production of specific mRNAs by RNA polymerase II (RNApolII). The mechanisms that underlie this regulation have been the subject of intense genetic, biochemical and computational studies[1–8]. The transcription start site (TSS) of a gene is the first nucleotide that is copied at the 5′ end of the corresponding mRNA. The region around a TSS is often referred to as the core promoter — which is required for recruitment of the transcription apparatus and can be thought of as the priming stage for transcription initiation. Owing to the strong link between TSSs and core promoters, the terms are often used interchangeably. A confounding issue is that many genes have multiple TSSs that are located in close proximity to each other. For clarity, we define the TSS as a unique nucleotide that will be the first to be transcribed, whereas the core promoter is defined as a genomic region that spans this and the nearby TSSs.

In the past, TSSs and core promoters have been identified on a gene-by-gene basis by one of two methods, nuclease protection or primer extension (BOX 1), neither of which can be applied on a genome-wide scale. Known TSSs are used to find *cis*-regulatory elements that are assumed to lie upstream of the TSS. This is frequently done by fusing the upstream sequence to a reporter gene and then introducing targeted deletions in that sequence to decipher where crucial elements reside. This approach is often referred to as a reporter gene assay, and underlies much of our knowledge of *cis*-regulatory elements.

Cis-regulatory elements of core promoters are commonly conserved across orthologous genes and contribute to the specificity of transcription initiation[1]. However, the complete set of mammalian promoters is too diverse to allow reliable computational annotation of genomic DNA sequence without reference to the experimentally determined locations of full-length cDNA sequences or TSSs from orthologous genes[8,9]. This limitation has motivated the development and application of high-throughput methods to experimentally identify TSSs and their flanking core promoters as a foundation for understanding transcriptional control, and as a tool for genome annotation. Results from these high-throughput studies have revealed a surprisingly large number of novel intergenic transcripts and promoters, prompting us to rethink mammalian promoter architecture.

Here we review recent relevant studies and technological developments to describe the current state of our knowledge of core promoter architecture and function, and highlight future challenges.

[#]*ARC Special Research Centre for Functional and Applied Genomics, Institute for Molecular Bioscience, The University of Queensland, Brisbane QLD, 4072, Australia.*
Correspondence to D.A.H.
e-mail: d.hume@imb.uq.edu.au
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Core promoter
The genomic region that surrounds a TSS or cluster of TSSs. There is no absolute definition for the length of a core promoter; it is generally defined empirically as the segment of DNA that is required to recruit the transcription initiation complex and initiate transcription, given the appropriate external signals (such as enhancers).

Orthologues
Genes that originate from the same ancestral gene and are diverged by a speciation event.

Mediator complex
A multi-subunit complex that can respond to many different activators (such as DNA-bound transcription factors) and links such signals to the core promoter and the transcription machinery.

Tag library
A tag library is similar to a conventional cDNA library, except that, subsequently to isolation and cloning of the cDNA, small fragments are generated by restriction-enzyme cleavage, concatamerized and recloned. This approach enables efficient DNA sequencing of thousands of tags from a single library.

RNApolII transcription initiation

The 'textbook' model of an RNApolII promoter has an AT-rich DNA sequence (the TATA box) approximately 30 bp upstream of an initiator (Inr) sequence that contains the TSS. Assembly of a pre-initiation complex (PIC), which includes the transcription factor TFIIA-H along with RNApolII, at such promoters is initiated by TFIID binding to the TATA box, Inr sequences and/or other sites, and bending DNA through a 90° angle. The next step involves recruitment of general transcription factors[2], after which transcription is initiated 30 bp downstream (see REFS 1–4,10,11 for reviews). Basal and regulated transcription initiation generally also involves interactions of the PIC with three additional components: the TATA-associated factors, the so-called mediator complex(es), and positive and negative cofactors. Coordination of chromatin modification, mainly through the control of post-translational modification of histones, also has an important role in transcription initiation[1–6]. The recruitment of all of these co-activators and co-repressors of transcription initiation is controlled by transcription factor binding to *cis*-acting DNA sequences that can lie within the core promoter or in more remote locations (enhancers and repressors)[8].

Apart from the TATA-box, subsets of promoters contain the Inr element, CpG islands and other sequence patterns[1] (BOX 2), but their prevalence and role in the initiation of transcription are not as well characterized.

It is now clear that TATA-driven PIC assembly is the exception, rather than the rule, in eukaryotic transcription, as only a fraction of mammalian promoters (10–20% (REFS 12,13)) contain a functional TATA box. Similar conclusions have been reached from genome-wide analysis of *Drosophila melanogaster*[14,15] and *Arabidopsis thaliana*[16] promoters. In fact, in mammals, TATA-containing promoters are commonly associated with tissue- or context-specific genes[17].

Genome-wide TSS discovery

The availability of the genome sequences of many eukaryotes has enabled the development of methods designed to analyse features such as gene boundaries, epigenetic effects and active *cis*-regulatory sites on a genome-wide scale[10,11,18–24]. A subset of these are targeted specifically towards locating the 5′ boundaries of transcripts or active TSSs[10,11,12,23–27] (BOX 3). Genome-wide approaches involve several sequencing-based high-throughput methods that require reliable isolation of full-length cDNAs, sequencing of their 5′ ends and mapping of the sequence to a completed genomic DNA sequence. The sequencing stage can use the 5′ ends of cloned full-length cDNA libraries (so-called 5′ ESTs[28,29]), short tags derived from 5′ ends of capped RNAs (CAGE[30] (cap analysis of gene expression; see the CAGE Basic Viewer and the CAGE Analysis Viewer web sites) and 5′-SAGE[30–33] (serial analysis of gene expression)) or tags derived from 5′–3′ ends (so-called paired-end tags (PET)[34]). All of these methods use reverse transcription to generate cDNA, and a full-length copy of the template RNA is not always produced. The key to accuracy is the stringency with which genuine full-length cDNAs are captured before sequencing. An important feature that distinguishes EST and full-length cDNA sequencing from the other tagging methods is the throughput, which is evident in the data sets that are already available. For the mouse, we have around 100,000 sequenced full-length cDNAs, around 1,000,000 5′-end sequences derived from full-length cDNAs, and more than 10,000,000 CAGE and other 5′-end tags. The depth of sequencing that can be achieved with tag methods means that the frequency of tags in an individual library can be used to indicate levels of expression. With new sequencing technology, tag sequencing might well supplant microarrays as a way of analysing gene expression.

In addition to the sequencing-based methods, another, albeit less precise, approach to identifying promoters involves chromatin immunoprecipitation (ChIP)[35,36] of DNA-bound, promoter-associated proteins, where the bound DNA is applied to tiling arrays known as ChIP–chip) (BOX 1). Alternatively, ChIP material can be used as a substrate for tag library construction, and the ChIP products can be analysed by sequencing[37,38].

Putative 5′-end regions can also be identified by hybridizing labelled cDNA or cRNA to high-density genome tiling arrays. Subsequent large-scale bidirectional cDNA synthesis using gene-specific primers is used to extend and validate the 5′ boundaries and identify promoter regions[19,22,39].

Scaled-up 'standard' labour-intensive molecular biology methods such as reporter gene assays and 5′ RACE on individual genes using gene-specific primers[40] are generally not feasible for whole-genome studies. Nevertheless, they have been used successfully for comprehensive screening of promoters in the 1% of the human genome that was chosen for intensive analysis in the first phase of the ENCODE (Encyclopedia of DNA Elements) project[12] (see the UCSC ENCODE web site).

All of the above approaches (the tag-based methods in particular) have identified a large number of TSSs and associated core promoters. Among them, only those that use PET ditags[34] or 5′–3′-end-paired EST reads[23,28,41] provide information about the length of transcripts that are produced from identified 5′ ends.

Author addresses

*The Bioinformatics Centre, Department of Molecular Biology & Biotech Research and Innovation Centre, Ole Maaløes Vej 5, DK-2100 København Ø, Denmark.
‡Genome Exploration Research Group (Genome Network Project Core Group), RIKEN Genomic Sciences Center (GSC), RIKEN Yokohama Institute, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045, Japan.
§Genome Science Laboratory, Discovery and Research Institute, RIKEN Wako Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan.
||Computational Biology Unit, Bergen Center for Computational Science, and Sars International Centre for Marine Molecular Biology, University of Bergen, Thormøhlensgate 55, N-5008 Bergen, Norway.
¶MRC Functional Genetics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 3QX, United Kingdom.

Box 1 | **Overview of hybridization-based methods for TSS identification**

There are two types of method for inferring transcription start sites (TSSs): those that are based on sequencing of cDNAs, and those that involve hybridization of RNA or cDNA to DNA probes. Some methods allow for targeting to a specific genomic region or gene, whereas others, by design, must be applied to whole genomes. Examples of hybridization-based methods are given below, listing the distinctive advantages and drawbacks (see BOX 3 for sequence-based methods).

Nuclease protection assay

Nuclease protection methods rely on hybridizing a labelled DNA probe, designed to be complementary to a postulated TSS region, with a source of mRNA, and incubating with a nuclease (often S1 nuclease) that cleaves single-stranded molecules[97]. The length of the protected fragment (inferred from gel electrophoresis) can be used to deduce the length of the mRNA and the corresponding position of the TSS on genomic DNA.

Keywords. Gel-based, low throughput, targeted.

Advantage. This technology is independent of reverse transcriptase reactions.

Disadvantages. Apart from the low-throughput nature of the method, the gel-based interpretation of transcript sizes (and therefore TSS locations) is difficult if there are many TSSs in close proximity. Also, controlling nuclease activity requires appropriate enzyme calibration, and this method requires the use of radioisotopes.

Primer extension

Primer extension analysis uses a labelled primer that is complementary to an internal region of an mRNA that is used for runoff reverse transcription of the mRNA template. The products are typically subjected to partial DNA digestion, following which the resulting labelled fragments are separated on a denaturing polyacrylamide gel and compared with DNA fragments of known size, typically a sequencing ladder. Variants of the primer extension method involve stringent capture of the 5′ end of the mRNA on the basis of the chemical modification (the cap) on the first base of mammalian mRNAs.

Keywords. Gel-based, low throughput, targeted.

Advantages. This method provides the length of the RNA starting from the primer in a similar way to the rapid amplification of cDNA ends (RACE) method but without amplification, and is less laborious than nuclease protection assays.

Disadvantages. It is low throughput and requires detection with radioisotopes. Compared with RACE, this method requires more starting material but shares all its disadvantages.

Tiling arrays

Regardless of the presence of the cap, RNA is randomly primed in order to minimize any possible bias caused by oligo-dT primers. Once double-stranded cDNAs have been synthesized, they are further fragmented with DNase I and extended with terminal nucleotidyl transferase, which adds biotinylated nucleotides. After hybridization to arrays, an antibody against biotin is used to detect signal (see figure).

Keywords. Hybridization-based, whole genome or targeted, often strandless.

Advantages. Tiling arrays provide a snapshot of all the transcribed regions in the genome, not only the 5′ or 3′ ends. This can be obtained in a single experiment at a fraction of the cost of full-length cDNA sequencing. Chips can be customized for chosen regions or can cover the whole non-repetitive part of the genome.

Disadvantages. This techinique must be complemented by RACE or tags/ditags to infer where the edges of exons lie, especially at the 5′ and 3′ ends. It integrates signals from all transcripts in a sample into a single signal, which means that alternative-splicing information and splicing patterns cannot be distinguished. Some platforms cannot distinguish which of the two DNA strands is being transcribed.

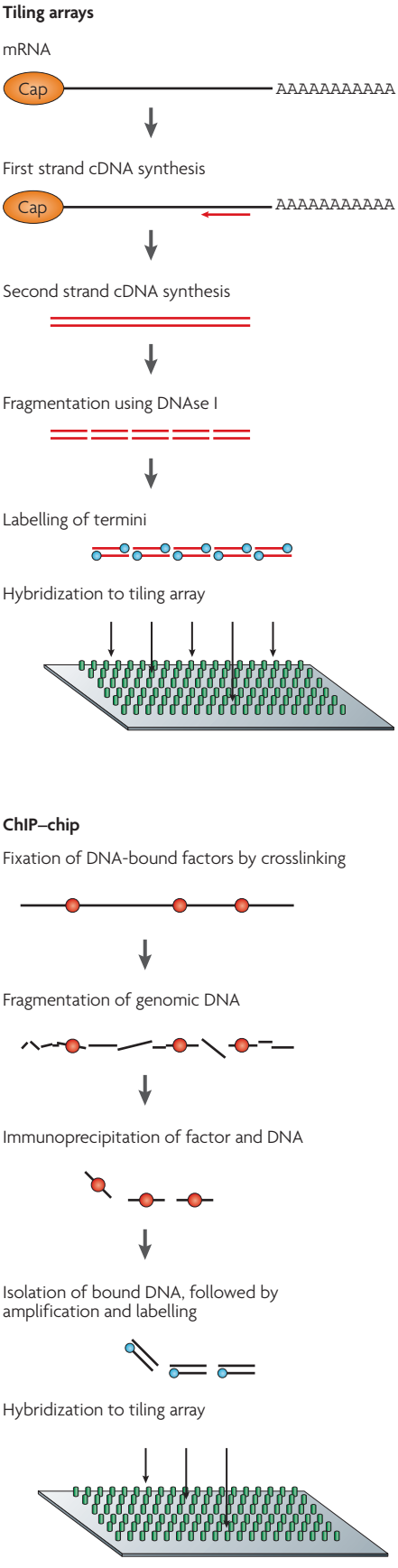
ChIP–chip

In chromatin immunoprecipitation (ChIP), antibodies are used to isolate DNA fragments that are bound to DNA binding proteins or their complexes. After crosslinking the whole proteins to the genomic DNA, the genome is reduced to fragments of a few hundred base pairs in length by sonication. Subsequently, an antibody that recognizes a specific nuclear protein of interest is used to isolate specific complexes. The DNA is then purified, and after ligation of appropriate linkers, it is amplified and labelled. Hybridization of the labelled DNA fragments to whole-genome tiling arrays reveals the genomic location of the DNA (see figure). Alternative protocols have used sequencing instead of DNA arrays.

Keywords: Hybridization-based, provides locations of *in-vivo* DNA-bound proteins

Advantages: ChIP–chip shares many of the features of tiling arrays (see above). However, a unique feature is that biologically active transcription factors or enzymes (such as RNApolII) can be captured 'in action'. Specific antibodies that are targeted to specific molecules can be used (such as phosphorylated RNApolII).

Disadvantages: The hybridization of relatively large DNA fragments after sonication to tiling arrays results in detection of a larger region of DNA than is actually covered by the DNA-bound protein. The results can easily be over-interpreted, as the method shows only the DNA binding site of the protein, but does not necessarily describe the actual function of the interaction.



Box 2 | **Common DNA elements in core promoters**

A set of common DNA sequence elements and patterns are associated with core promoters. These patterns have important characteristics that are linked to the expression of the downstream genes. Different elements can co-occur in the same promoter, although certain combinations are more likely than others, and some patterns complement each other. Below we provide a brief overview of the best studied elements (for a more comprehensive discussion, see REFS 1,2,7). Analysis of the locations of these elements relative to experimentally defined transcription start sites (TSSs) is provided in FIG. 2. For description purposes, the patterns are presented as International Union for Pure and Applied Chemistry (IUPAC) consensus symbols: N, any nucleotide; R, A or G (purine); S, C or G; V, A or C or T; W, A or T; Y, C or T (pyrimidine).

TATA box

The TATA box, located 28–34 bp upstream of the TSS, is perhaps the best known transcription factor binding site. Its consensus sequence, TATAA, binds the TATA-box binding protein (TBP), which is part of the pre-initiation complex (PIC). TATA boxes are associated with strong tissue-specific promoters, and often co-occur with initiator (Inr)-like sequences at the initiation site. Binding of TBP to the TATA box enforces the PIC to select a TSS in a limited genomic space.

Initiator element

The Inr element, defined by the YYANWYY consensus where the A is at position +1 (REF. 1), is independent of the TATA box, although the two can occur together and act synergistically. The TATA and Inr elements are the only known core promoter elements that, alone, can recruit the PIC and initiate transcription.

Downstream promoter element (DPE)

The DPE lies 28–32 bp downstream of the TSS in TATA-less promoters of *Drosophila melanogaster*[15,98]. It has a RGWYV consensus. Generally, it occurs together with Inr elements. The DPE is thought to have a similar function to the TATA box in directing the PIC to a nearby TSS[98].

TFIIB recognition element (BRE)

The BRE element, with an SSRCGCC consensus, lies upstream of the TATA box in some TATA-dependant promoters[99]. It can either increase or decrease transcription rates in eukaryotes, although the details of this meachnism are unknown.

CpG island

CpG islands are genomic stretches in which CG dinucleotides are overrepresentated[100]. On the basis of the original computational definition of CpG islands, 50% of human promoters are associated with CpG islands[101]. Subsequent studies using a statistically derived definition of CpG islands increased this fraction to 72%[102]. CpG-island-associated promoters are most often associated with so-called housekeeping, or ubiquitous, genes[17], although there are many exceptions, including brain-specific genes[103]. Only a fraction of CpG-associated promoters have TATA-like elements.

The most extensive core promoter identification study undertaken so far used CAGE tags to identify 184,379 human and 177,349 mouse core promoters, many of which might contain a cluster of individual TSSs[24]. A previous analysis that involved full-length cDNA sequencing identified 30,964 human and 19,023 mouse promoters[42]. But even the most recent figures are likely to be a substantial underestimate. First, sequencing 50–100,000 tags in each library can reliably detect only those transcripts that are expressed at a level of at least 10 copies in each cell (as there are at least 400,000 mRNAs in an average mammalian cell[43]). Many transcripts are not present at this level, either because they are of low abundance in individual cells or are expressed in only a small subset of cells in the tissues that have been studied.

The second source of underestimation derives from the fact that some CAGE tags cannot be mapped to a single genomic location. A substantial fraction of these map to two or three regions, perhaps indicating that core promoter sub-sequences are shared by distinct promoters. It might be possible to resolve them by merging CAGE data with the extensive collection of 5′ ESTs and PETs. The remaining multi-mapping tags map to repeat regions[24]. There is evidence of promoter activities residing in long terminal repeats of transposons[44,45], but which of these elements are active remains unknown.

The above CAGE-based study focused only on TSSs that were detected more than once (with two or more tags mapping to the same site) and that mapped unequivocally to one genomic location, although many lines of evidence indicated that the large majority of singletons were also true TSSs[24]. The data were validated by various experimental and statistical methods, including RACE, comparison to known full-length cDNA sequences, and conservation of precise TSSs between human and mouse orthologous genes[24]. They are also compatible with independently derived human data sets, which were assembled mainly using oligocapping methods and from full-length cDNA collections that are present in the Database of Transcriptional Start Sites (DBTSS)[42].

Overall, genome-wide approaches, including cDNA sequencing and tiling array studies, reveal a surprisingly large number of novel, intergenic transcripts and promoters[19,22–24]. Many newly identified promoters are not associated with identifiable downstream ORFs, and thus probably direct production of non-protein-coding RNAs. Several novel promoters lie in regions that were thought to be gene deserts on the basis of mapping of full-length cDNAs[19,22–24,39]. This might be correlated with the observation that transcription factor binding sites are often distant from a known gene[18]: there are examples of crucial distal enhancers that are transcribed[46–48], producing non-coding RNA (ncRNA) products that can function in imprinting[46] or as transcriptional co-activators. Given their abundance and apparent diversity, it is thought that many other roles of ncRNAs remain to be discovered.

Box 3 | **Overview of sequencing-based methods for TSS identification**

The methods for inferring transcription start site (TSSs) are either based on sequencing of cDNAs, or rely on hybridization (see BOX 1). Descriptions and distinctive advantages and drawbacks for sequence-based methods are listed below.

RACE

Rapid amplification of cDNA ends (RACE)[40] is used to detect the 5′ ends of individual RNAs. It can be thought of as a 'next-generation' primer extension protocol. RNA is at first oligo-capped; a phosphatase treatment removes the phosphate groups from truncated or uncapped RNA molecules, whereas full-length mRNAs remain protected by the cap structure. Subsequently, the cap is removed by tobacco acid pyrophosphatase, leaving a 5′-end phosphate group that is used by RNA ligase to conjugate an oligonucleotide to the 5′ end. For specific transcripts, primers (or a set of nested primers to increase specificity) are then used for a reverse transcription (RT)-PCR reaction. The product is cloned and sequenced (see figure).

Keywords: Sequencing-based, low throughput, targeted

Advantages: RACE is highly sensitive and complements 5′ tagging. This method is useful for targeting particular loci of interest with higher scalability than hybidization-based approaches, making it ideal for verifying particular TSSs that have been identified by high-throughput methods.

Disadvantages: It is a low-throughput method, as different primers should be designed to validate each TSS in separate experiments. Similar to 5′ tagging methods, information about the span of the full transcript is not retained.

5′ tag sequencing

5′ tag sequencing[25], exemplified by the cap analysis of gene expression (CAGE) technique, allows high-throughput identification of TSSs. Cap-trapping ensures that only the cDNA molecules that are extended to the (biotinylated) cap site are selected; cDNAs that fail to reach the cap site are not selected, because RNase treatment removes the biotinylated cap from the partial cDNA–mRNA hybrid molecule. A linker that contains an *Mme*I restriction site is then ligated to the 5′ end of cDNAs. After the second strand synthesis, *Mme*I cleaves 20–21 nucleotides within the cDNA, producing a 5′-end tag. After ligation with another linker and PCR amplification, tags are concatenated and cloned in a plasmid vector. Concatenation enables sequencing of multiple tags in a single run, decreasing costs (see figure). Sequenced tags are then mapped to the genome using alignment programs such as BLAST[104].

Keywords: Sequencing-based, high throughput, genome-wide only

Advantages: These methods have the highest throughputs. A unique feature is that both TSS location and degree of usage (the number of tags mapping to a specific location is roughly proportional to the transcription level) can be determined. Using material from many different tissues, tissue-specific promoters can be located.

Disadvantages: The short span that is covered by the tags make some alignments unusable (for instance, mappings of transcribed repeat elements). Information about the span of the full transcript is not retained.

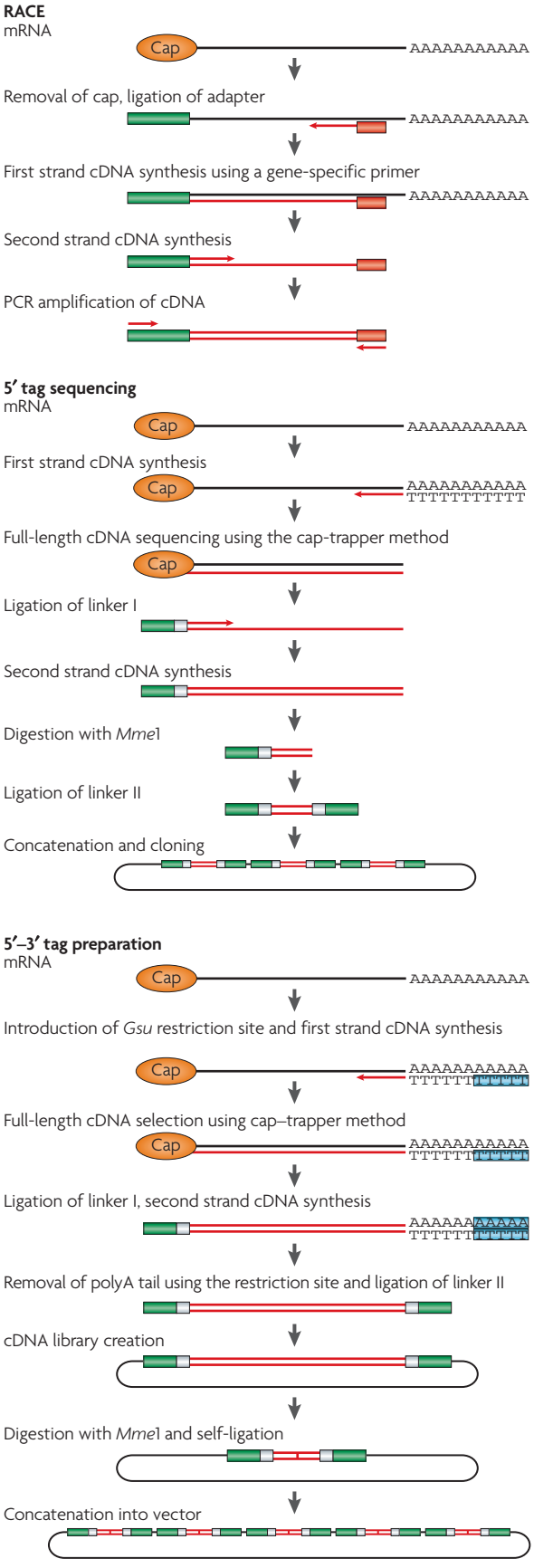
5′–3′ tag sequencing

Exemplified by paired-end ditag technology (PET), a full-length cDNA library is first prepared using the cap-trapping method. An oligo-dT primer is used to prime the first strand cDNA synthesis. The oligo also carries a *Gsu*I restriction site, which enables the removal of the 3′ polyadenylated stretch following restriction with *Gsu*I. The cap-trapped, deadenylated full-length cDNA is ligated to a linker at the 3′ end, which is used for subsequent cloning into a plasmid vector. As there are *Mme*I sites at both cDNA ends, *Mme*I cleavage removes the cDNA insert, except for the 5′–3′-end tags. After re-ligation, the 5′–3′ ditags are excised again and used to produce concatamers (see figure).

Keywords: Sequencing-based, high throughput, genome-wide only

Advantages: Sequencing of both 5′ and 3′ ends makes assigning TSSs to transcripts less problematic. The method gives additional information because the 3′ UTRs can harbour important *cis*-regulatory elements. It can also be used together with ChIP to sequence DNA that is bound by a factor of interest.

Disadvantages: 5′–3′ tag sequencing is a lower-throughput method than 5′-end tagging. As there are more steps in the protocol, the risk of introducing bias increases during the numerous DNA amplification steps, which might cause the information that can be derived from long mRNAs to be lost.



The integration of TSS, transcription factor binding data and phylogenetic information from multiple mammalian genomic sequences has the potential to identify new classes of genome regulatory elements in regions we currently regard as 'intergenic'. Even with our knowledge to date, the conceptual framework of a genome that is simply organized into distinct gene units must be revised[49]. What emerges instead is a transcriptional landscape in which there are no obvious boundaries between the units we currently call genes[23].

Redefining the anatomy of core promoters

The CAGE-based approach indicated that most human and mouse promoters lack the distinct TSS that is commonly assumed to be located at one specific genomic position; instead, the typical core promoter architecture consists of an array of closely located TSSs that spread over around 50–100 bp[23,24]. Many hybrids between these two types of promoter also exist; for instance, in some promoters, TSSs are distributed over a large region, but most transcription initiates at one specific nucleotide position. These observations provide the basis for a new system of promoter classification — it seems more relevant to describe promoters using a TSS distribution that shows the preferred initiation-site usage in a genomic window, instead of a static single position (FIG. 1).

The mapping of smaller datasets of 5′ ends of cDNAs[50] and in-depth studies of individual CpG-enriched promoters (reviewed in REF. 1) have also identified broad TSS regions. Adding further support to this classification, orthologous human and mouse promoters share strikingly similar profiles of TSS usage (FIG. 1). Although a finer subclassification of promoters on the basis of TSS distribution has been proposed[24], for simplicity, we here refer to any of the broad TSS classes as 'broad' and the single TSS groups as 'sharp'. In general, the second classification correlates with the presence of a TATA box, as this feature is associated with promoters that have a single, sharply defined TSS (FIG. 2). The prevalence of distinct TSSs in TATA-box-containing promoters has also been confirmed by analysis of smaller promoter sets[50]. However, not all sharp promoters have a TATA-box (FIGS 1,2), and it will be interesting to examine these cases in detail in future.

Whereas TATA boxes are mostly found in sharp promoters, CpG islands are overrepresented in broad promoters (BOX 2). Sharp promoters are primarily used for tissue-specific expression, whereas broad promoters are generally associated with ubiquitously expressed genes, which is also true for promoters that are associated with TATA boxes and CpG islands, respectively (BOX 2).

Statistical analysis[51] and structural studies[52] indicate that TATA-box position can vary 28–34 bp from the first T in the TATAA consensus to the major initiation-site peak, with a distance of 30–31 bp being strongly preferred. These observations also agree with experimental evidence that shows that, if the TATA site is moved outside these boundaries, new initiation sites that correspond to more favourable TATA–TSS distances arise[53–55]. Ponjavic *et al*[51] showed that the tissue specificity of the promoter is generally highest if the TATA box is located at –30 bp or –31 bp relative to the dominant TSS, and that the initiation-site consensus varies with the TATA–TSS distance. This and another study[16] indicate that longer TATA–TSS distances (32–34 bp) are used more frequently than the shorter ones (28–29 bp). The selection of more distant TSSs seems to be driven by the lack of strong initiation sites (see below) at a more favourable distance.

As noted above, as the number of mammalian promoters that have beeen analysed increases, the proportion of these that contain TATA boxes has decreased. This trend probably reflects the preference among the pioneers of mammalian promoter analysis towards studying highly expressed, tissue-specific genes. This fraction is likely to fall further as more data become available; given the current data coverage, the selection of promoters to study remains biased towards highly expressed genes.

Precise transcription initiation in TATA-box-containing promoters generally requires both the TATA box and an Inr-like element[54]. In a small subset of TATA-less promoters, the Inr sequence alone seems to be able to direct initiation at a single precise location[56,57]. However, most promoters, regardless of class, lack the classical Inr consensus sequence (FIG. 2). Even in its absence, the precise start point of RNApolII-mediated transcription is not random. The [–1,+1] dinucleotide relative to the initiation site shows strong conservation over the whole set of core promoters (a pyrimidine–purine (PyPu) consensus)[24]. The requirement for a purine at +1 is more stringent, but the reported strict requirement for A as a start position[1] is not universal.

Despite the minimal sequence constraint, the importance of the dinucleotide initiator is evident from evolutionary studies. Comparing tag frequencies in orthologous mouse and human promoters reveals that gain or loss of the TSS between species correlates with the creation or removal of the PyPu initiation site[24], respectively. The initiation-site preference varies with initiation-site usage. Highly used TSSs tend to use CG, TG and CA dinucleotides, whereas rarely used TSSs diverge from the preferred PyPu dinucleotide, particularly favouring GG[24]. An example of this can be seen in FIG. 1Bb, in which most of the larger TSS peaks lie over CG, TG or CA sites. The key role of the initiator dinucleotide is supported by a recent study of the ankyrin 1 promoter. In a human patient, deletion of a TG dinucleotide that is used as a TSS in this gene was associated with reduced promoter activity and abolition of TFIID binding to that particular TSS[58].

One problem with using multiple start sites over an extended genomic region is that translation generally starts with the first ATG in an mRNA. Broad promoters must therefore exclude ATG start codons from a certain region, so that all mRNAs that are generated can be efficiently translated. In support of this, a recent study of MHC class I genes identified a set of TATA-less and Inr-less promoters that have multiple TSSs and exhibit such a depletion of ATG trinucleotides in the promoter region[59]. Using orthologous promoters in human, mouse and rat genomes, the authors estimated

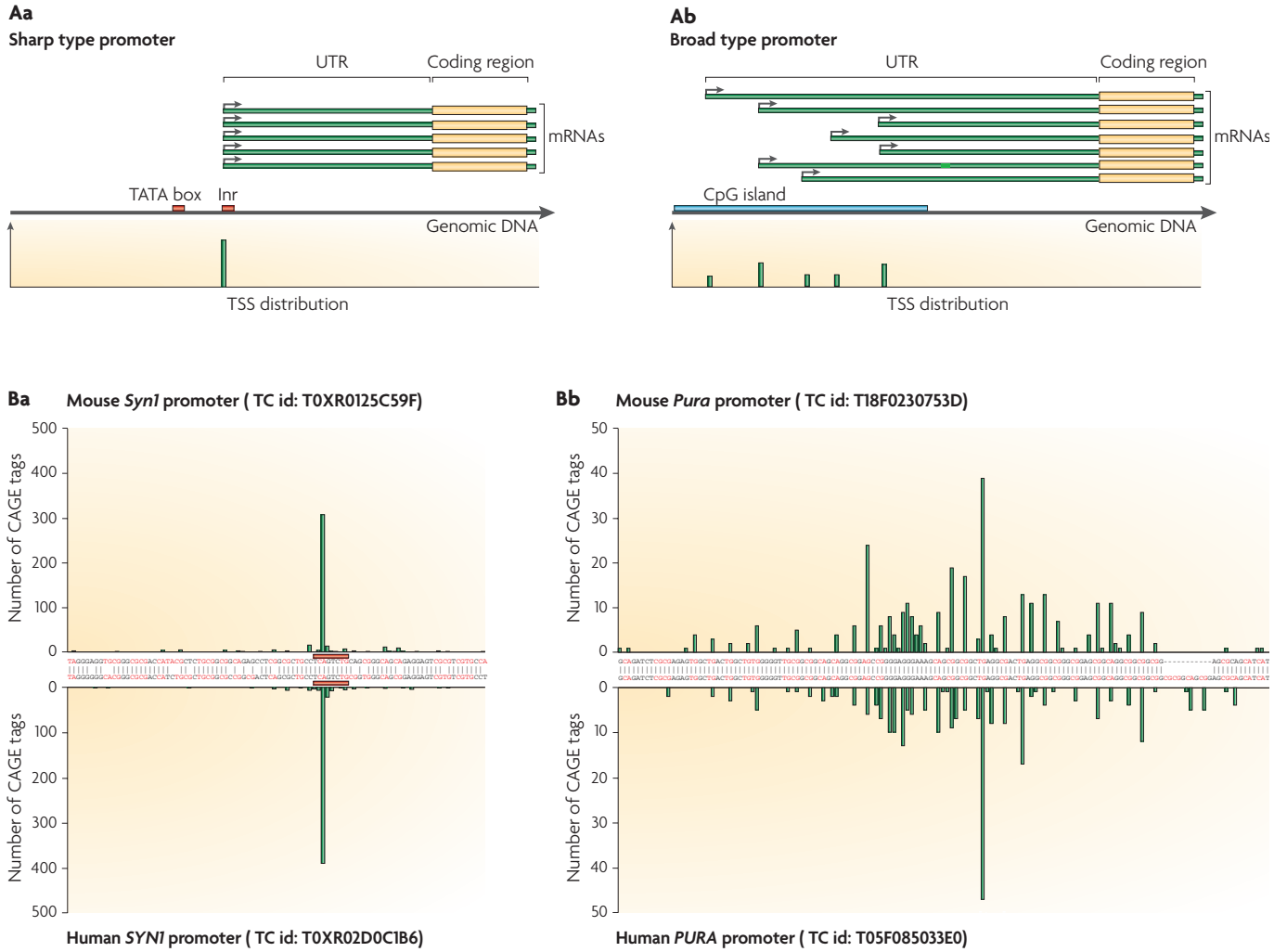


Figure 1 | **TSS classes in mammalian promoters.** Promoters can be classified with respect to the distribution of the transcription start sites (TSSs) they use. For each class, we first show the general features of the class (part **A**) and then a detailed example (part **B**). For these examples, the CAGE (cap analysis of gene expression) tag distribution of the TSSs in the mouse genome (top panel) and of the orthologous TSSs in the human genome (bottom panel) is shown on the *Y* axis. The *X* axis shows the alignment positions between the mouse and human promoter region. Pyrimidine–purine (PyPu) dinucleotides are coloured red — note the correspondence to the major initiation-site peaks. **Aa** | Promoters that fall into the 'sharp' class use only one or a few consecutive nucleotides as TSSs, resulting in a single-peak TSS distribution. These promoters often have TATA and initiator (Inr) boxes. **Ab** | Promoters that fall into the 'broad' class can initiate transcription over a ~100 bp region, resulting in a population of mRNAs that have different lengths but usually the same protein-coding content. Broad promoters are often TATA-less and CpG-island-enriched. **Ba** | The single-peak promoter class is exemplified by the *Syn1* gene, in which almost all TSSs are concentrated in a few consecutive positions, consistent with textbook models of promoters. This particular promoter has a distinct Inr sequence that directs transcription (indicated by a red line), but no obvious TATA box. **Bb** | The *Pura* gene promoter consists of TSSs that are spread over a larger genomic space. Most of the main TSSs in this promoter have a PyPu dinucleotide, but there are no clear Inr consensus sequences. Note the corespondance between human and mouse TSS usage.

that ~82% (6,595 of 8,003) of human genes have 'ATG-desert' characteristics. Collectively, these new data force us to re-evaluate our understanding of transcriptional initiation. The data imply that the TFIID complex, which is essential for all classes of RNApolII transcription, actually binds relatively non-specifically, without an absolute preference for promoters with a TATA box or the strong Inr-like element, and scans along the DNA for a TSS[60].

Tag cluster
This Review defines tag clusters as genomic regions in which two or more tags (of 20 nucleotides in length) overlap each other (both being mapped to the same strand).

The size of core promoters

Studies of TSS usage with genome-scale approaches have provided an overview of the characteristics of broad promoters as a class. The median genomic span that is covered by CAGE tag clusters in broad promoters is 71 bp, and is seldom larger than 150 bp (94.3% of broad promoters that were assessed are smaller than 150 bp when considering the minimum nucleotide range that contains 75% of tags in a cluster).

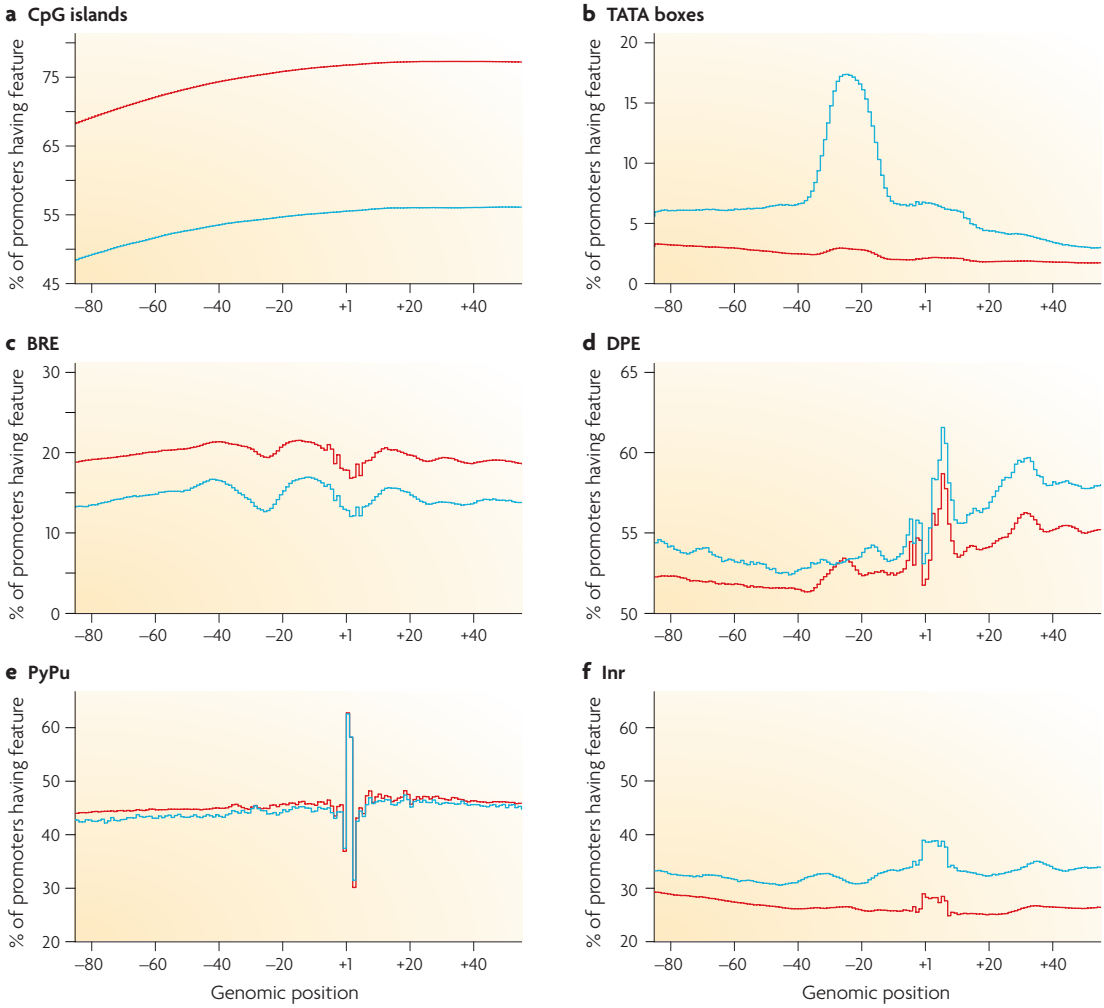


Figure 2 | **DNA motif predictions in core promoters depends on TSS architecture.** Transcription start site (TSS) clusters from a CAGE (cap analysis of gene expression) study[24] that used more than 100 tags from mouse were divided into sharp (indicated in blue) or broad (indicated in red) TSS distribution classes. For each TSS position in the clusters, the –80 to +40 promoter region was scanned using matrix models[8] for core promoter elements and analysed for CpG-island overlap, and the fraction of nucleotides in each position that belonged to the pattern in question was calculated (see Supplementary information S1 (box) for methodology). It is important to note that the absolute frequencies of detected sites are strongly dependent on the cutoffs that are specified in the model, although the aim here is to highlight the contrasts between different types of core promoter. **a** | The broad promoter class has substantially higher CpG coverage than the sharp class, agreeing with previous results[24]. **b** | Consistent with panel **a**, the sharp class has a higher fraction of TATA-matching nucleotides, which are concentrated at the expected location (around –30 to –22). With the cutoff value used here, only around 17% of the sharp class promoters have a canonical TATA site. Even if the fraction was twice as high, this is significantly lower than expected on the basis of the earlier belief that most promoters have a TATA box (see main text); the sharp class of promoters is estimated to cover only about 25% of all promoters. **c** |The BRE (TFIIB recognition) element is reported to occur primarily in TATA-box promoters, just upstream of the TATA box. Surprisingly, this element occurs more often in broad-class promoters (which have significantly fewer TATA sites). This could be due to the higher GC content that is generally observed in broad-class promoters. Although the GC content is increased at the expected location (–37 to –32), a similar increase occurs at the –20 to –1 region. As this increase is modest compared with the background level, it is possible that BRE elements are used less in mammals than in *Drosophila melanogaster*, in which it first was discovered. **d** | The downstream promoter element (DPE) has been reported to lie in the +28 to +32 region in TATA-less promoters in *D. melanogaster*. At this position, the element is frequently observed in both promoter classes, but is most evident in the sharp promoter class, which is consistent with its ability to act as a substitute for the TATA box in directing the precise TSS selection. It is currently unclear whether the DPE is important in mammalian promoters. **e** | The number of pyrimidine–purine (PyPu) dinucleotides is approximately equivalent between the two classes at the actual TSS, but is higher for the broad class in the regions that flank it. This is primarily due to the large number of nearby TSS locations. **f** | The results in part **e** are in sharp contrast to the classical initiator (Inr) element, which is used less often than PyPu dinucleotides in both classes and is most prevalent in the sharp promoter class. The location of the Inr elements is consistent with previous knowledge, indicating that a subset of both classes use the Inr motif instead of the more ambiguous PyPu dinucleotide.

This width constraint is probably related to the length of DNA that is wrapped around a nucleosome and that of the linker DNA, which is ~150 bp[61]. Human promoters have been shown to be nucleosome free at the actual TSS region[62]. If the actual TSSs within such a nucleosome-free region are determined only by the ability of the TFIID complex to bind with some preference to PyPu dinucleotides, as discussed above, the promoter activity of broad-class promoters would be determined solely by the precise position of the nucleosomes, which in turn is regulated by post-translational modification of histones (see REFS 63,64 for reviews). Within these regions, the role of known core promoter elements such as the TATA box would be to restrict the TSS selection process to specific nucleotides.

Interestingly, Segal *et al.*[65] have recently provided evidence for the existence of nucleosome-positioning signals in the vicinity of yeast promoters, suggesting that there is an intrinsic tendency for promoters to exclude nucleosomes. Nevertheless, Kawaji *et al*[66] showed that some broad promoters have overlapping but distinct TSS distributions in different tissues, proving that TSS selection within the promoter can be regulated.

As part of the ENCODE project, a recent study used full-length cDNA mapping and reporter gene assays in 16 cell lines to validate 642 human promoters[12]. The study showed that deletions in the −350 to −40 region upstream of TSSs resulted in decreased reporter gene signals; this region roughly corresponds to the region that is conserved between the promoters of orthologous genes in mice and humans (see REF. 67 and below). The actual crucial interval is probably smaller in any one promoter, as in this study a single arbitrary TSS was chosen as the reference point (so parts of broad TSS regions were probably removed in a subset of cases), and many of the studied intervals contained more than one independently regulated promoter (see below). Focusing on small proximal promoter regions, taking into account the broad TSS regions, should expedite the computational identification of functional motifs that are conserved among classes of genes with common regulatory patterns.

Bidirectional promoters

Previous frameworks for promoter analysis frequently assumed that genes, and therefore their promoters, occupy distinct, non-overlapping genomic regions separated by non-functional DNA. Early studies identified one of the exceptions to this 'rule'[68–70]: genes that lie on opposite strands with their TSSs lying in close proximity to each other form so-called bidirectional promoters. Trinklein *et al*[71]. estimated that 1,352 gene pairs in the human genome have TSSs on the opposite strand that are separated by less than 1 kb; the corresponding number in the mouse was estimated to be 1,638 (REF. 72). Genome-wide analyses that take account of the many newly identified non-coding RNAs and CAGE tags have revealed that promoter overlap of this kind is even more common[23,24,73]. In the large majority of bidirectional promoters, the TSS distribution is of the broad type, although each promoter in a bidirectional pair has independent core promoter elements (the TSS distributions in the two directions generally do not overlap)[24]. In fact, Trinklein *et al*[71] reported that 23% of bidirectional pairs produce sense–antisense pairs that overlap at their 5′ ends, where the TSS region of one gene is upstream of the TSS region of its partner; CAGE data analysis suggests that this is an underestimate[24]. Hence, many bidirectional promoters might be more appropriately referred to as anti-directional or opposing promoter pairs.

Widespread alternative promoter usage

Most mouse and human protein-coding genes are associated with more than one promoter region[12,24,27,42,74]. These alternative promoters are generally used in different contexts or tissues, or to produce distinct protein products. In many cases, the different promoters generate alternative 5′ exons that might or might not contain alternative start codons, and that often splice into a common second exon. The same locus can be associated with both sharp and broad promoters or with multiple broad promoters, each of which has its own ATG desert. For example, the UDP-glucuronosyltransferase locus has at least seven promoters with different tissue expression profiles, each of which produces an alternative first exon and generates a distinct amino (N)-terminal sequence[12,24]. Similarly, the gelsolin gene (*GSN*) can be transcribed to produce a secreted plasma protein that functions as a scavenger of actin filaments or as a cytoplasmic regulator of the cytoskeleton, each from distinct macrophage- or liver-specific promoters, respectively[24]. It is generally accepted that alternative promoter use substantially contributes to the complexity of the mammalian proteome[23,24,73].

Alternative promoters within 3′ UTRs. Most well-supported alternative promoters are found either at the 5′ ends of known cDNAs or in protein-coding exons. However, Carninci *et al.* revealed clusters of TSSs in the last 20% of the terminal exons of protein-coding genes (mostly in 3′ UTRs), on the sense strand of the transcript[23]. At least 1,000 mouse transcription units are associated with at least one such TSS, and the associated core promoters are sufficient to drive transcription[24]. The function of such promoters and their corresponding transcripts are unknown. According to one hypothesis, some of the resulting transcripts will overlap with downstream genes on the other strand, forming potential *cis*-antisense pairs that could contribute to coordinated expression of neighbouring loci[23].

Weak alternative exonic promoters. Brodsky *et al.*[10] analysed the locations of active DNA-bound RNApolII in HeLa cells. Surprisingly, they found that RNApolII sites were concentrated preferentially in exons. The density of RNApolII sites in exons varied between genes, but did not correlate with mRNA levels. The authors attribute these unexpected results to a possible slowdown or pausing of RNApolII elongation within exons[75]. Indeed, an earlier study showed that variations in the speed of RNApolII elongation will affect the usage of splice sites[76]. Accordingly, the amount of exonic RNApolII sites was

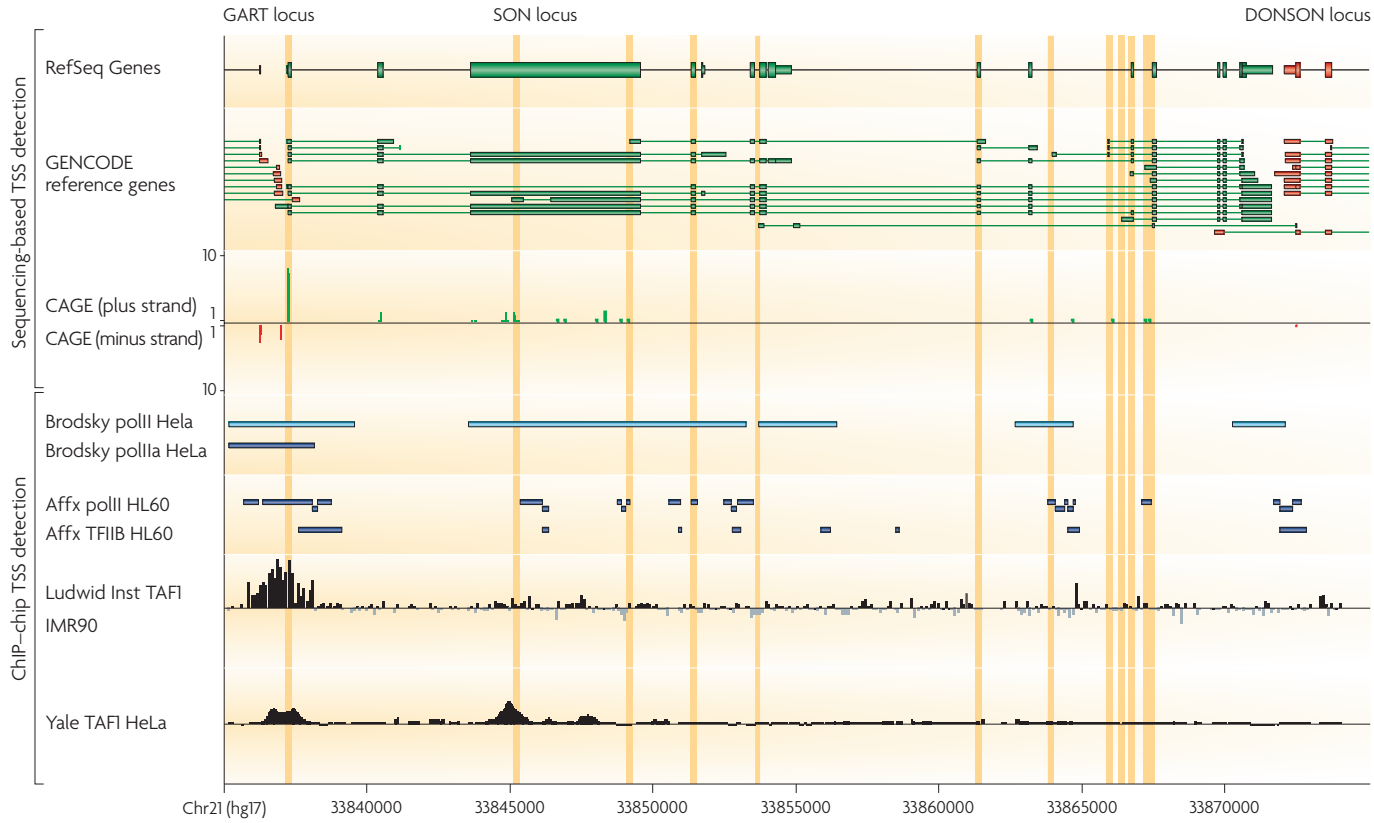


Figure 3 | **Complex TSS distributions within exons.** An example of exonic transcription start sites (TSSs) detected within the ENCODE[105] Enm005 region (human assembly hg17) using multiple techniques, on the basis of the UCSC ENCODE genome browser[96] representation, is shown. The RefSeq[74] track is collapsed owing to space limitations. The strand of the cDNA-based data is indicated in red (reverse) and green (forward). The GENCODE[107] track represents high-quality manual gene annotations from cDNA and EST sources, validated by RACE (rapid amplification of cDNA ends). TSSs within the SON locus, as indicated by GENCODE annotations, are highlighted with yellow columns. The results of CAGE (cap analysis of gene expression) tag sequencing and four distinct chromatin immunoprecipitation (ChIP)–chip experiments are shown as separate tracks below. All ChIP experiments except the Brodsky polII track were made using antibodies targeted to the pre-initiation complex. Many TSSs exist within RefSeq exons, as indicated by CAGE, GENCODE annotation and ChIP–chip. The positions of the majority of GENCODE TSSs that are located within RefSeq exons are supported by one or more of the other technologies; moreover, CAGE data indicate there are additional TSSs that are not detected by GENCODE annotation.

greater in alternatively spliced exons compared with the invariantly spliced ones[10].

A more provocative, although not mutually exclusive, explanation is that many of these RNApolII signals are due to genuine but infrequent initiation events, indicating that there are TSSs scattered within exons. In fact, many low-intensity exonic TSSs were found using CAGE tags[24]. The number of exonic TSSs varies between genes, (for example, exonic TSSs tend to be more prevalent in tissue-specific genes[24]) and this level is conserved between human and mouse orthologous genes. These observations suggest that truncated internally initiated mRNAs constitute a significant class of non-coding mRNAs; this is also consistent with evidence that the initiation complex can bind to sequences within exons but not introns[11] (FIG. 3). The possible function of weak initiation sites within internal exons requires further study, but it could contribute to the recently described phenomenon of exon-tethering — a physical connection between emergent splice sites in pre-mRNA and the RNApolII transcription complex[77].

Evolution of core promoters

Mapping of TSSs to a base-pair resolution on a global scale, together with reliable genome-wide alignments[78], have opened new avenues in promoter evolution studies. Promoter evolution among primate species occurs rapidly through accumulated substitutions and deletions[67,79]. An in-depth comparative evolutionary analysis between primate, mouse, rat and dog core promoters[67] revealed that the substitution rate at each site is lowest in the –50 bp to –1 bp region relative to the dominant TSS, and increases linearly until around –200 bp.

On the basis of the mouse–human comparison, TATA-box-containing promoters evolve more slowly than CpG-island-containing promoters[67]. This finding agrees with an earlier study that showed that context-specific genes have more conserved promoter regions than

other genes[80]. The implication is that the constrained and precise architecture of the TATA-box promoter is needed to ensure reliable transcription initiation in time and space; any change in the functional promoter sequence is likely to have a strong phenotypic consequence. In keeping with this view, a recent study of four closely related yeast species showed that TATA-box-containing promoters have a higher expression profile divergence between species than other promoters[81]; this was also observed in mammals, insects and plants[81]. Slower evolution at the sequence level of TATA-box-containing promoters but faster evolution in expression profiles between species is counterintuitive, but might reflect the fact that such promoters have only one TSS, making the initiation rate much more sensitive to any changes. By contrast, the broad core promoters with their multiple redundant TSSs respond to most mutations by smaller changes in expression, enabling fine-tuning of their activity and facilitating both adaptive evolution and compensation for mutations in the unfavourable direction[24].

The future of promoter analysis

Genome-wide analyses have identified the TATA-less promoter with multiple TSSs as the major class of mammalian promoters, clarified the role of the initiation site, identified sequence constraints in promoter regions, and facilitated more focused studies on the evolution and function of specific proximal promoter elements. They have also identified new classes of promoters that lie within exons and 3′ UTRs, and revealed the widespread use of alternative promoters in protein-coding loci and the functional organization of bidirectional promoter regions. These findings should drive future studies into the precise biochemical mechanisms of transcription initiation in the absence of the TATA box.

The precise location of TSSs enables a focused analysis of *cis*-acting elements that are bound by transcription factors. For example, Carninci *et al*.[24] were able to segregate promoters on the basis of their relative use in different tissues and cell types, and to show that, as would be expected, tissue-specific promoters are enriched for particular motifs that serve as the binding sites for known tissue-specific transcription factors. This analysis provides a starting point for unravelling the molecular details of cooperative interactions among transcription factors[82].

An important future challenge will be to reliably integrate TSS location data with related functional data, such as histone methylation and acetylation states[83,84], the position of nucleosomes[62,85] and the occupancy of transcription factor binding sites[38,86], each of which can now be contemplated on a genome-wide basis[36]. The linking of transcription factor binding sites that are not proximal to any gene with TSS selection for a particular promoter will present another important challenge.

It remains difficult to associate the 5′ ends of genes with their corresponding transcripts, especially if the TSS is novel. Some intragenic TSSs produce transcripts that bridge two or more downstream genes[87] but it might not be biologically relevant to associate these TSSs with the protein-coding gene in which they occur for other non-standard promoter types (3′ UTR promoters in particular).

If there are no cDNAs in a sample that map to the region in which a TSS lies, tiling array data might help to detect nearby transcribed regions (presumed to be exons). However, there is no reliable way of assigning an exon structure or TSSs to transcribed fragments that have been identified by tiling arrays without using full-length cDNAs or extensive cloning of 5′–3′ RACE products.

Chromosome conformation capture is a promising method for linking TSSs and regulatory elements to their corresponding transcript. This technique detects the physical interactions between chromosomal regions that are involved in common regulatory mechanisms[88–90]. This type of data integration is necessary if we are to understand the interaction between regulatory elements and regulatory proteins, and how they function together to direct transcription. Large-scale TSS data is crucial but not sufficient in itself for making this leap.

On a functional level, the next generation of experiments should consider the extent and function of novel transcripts in normal cells, instead of the actively proliferating cells that most ChIP–chip and tiling array data currently come from. Because proliferation requires specific transcriptional programmes, many observed transcripts might encode molecules that are specific to these processes. Despite the success of the genome-wide studies so far, the coverage of TSSs is incomplete; similar methods should be applied to other species, as only two mammalian species (mice and humans) have been investigated in any detail. High-throughput studies should also be extended to different tissues, as properties of promoters vary substantially between different contexts, tissues and cell types.

The multitude of newly found promoters is an obvious boon for computational biologists. The tag-based methods in particular provide both quantitative and nucleotide-positional information on tissue specificity, thus removing the most serious bottleneck for defining tissue-specific *cis*-regulatory elements[91].

For experimental biologists, the genome-wide data sets provide a valuable map of the promoter regions of genes of interest to guide further studies. The depth of data for mice and humans allows functional alignment of promoters[92] in phylogenetic studies as more complete mammalian genome sequences become available. As both tag and tiling array approaches and their related technologies reach maturity, we can expect the generation of genome-wide data sets to increase rapidly; for example, massive parallel sequencing technologies[21,93,94] are being integrated into pipelines for tag sequencing, and new applications of tiling arrays, such as hypersensitive site detection, are appearing[20,95]. The increasing depth of data to describe promoter architecture and diversity leads to numerous hypotheses about the function of motifs and motif combinations that must be tested experimentally. The pilot phase of the ENCODE project[96] and its extension to a larger part of the human genome will be a driving force for assessing new technologies and deepening our understanding of the transcriptome and its regulation.

Conclusions

Large-scale studies re-emphasize the take-home message that has emerged from both old and recent reviews of promoter function, and that has been largely ignored in computational studies: mammalian genomes harbour many types of core promoter. It is now evident that the rules for start site selection are fundamentally different for different promoters, and large-scale studies have given us the tools to partition promoters into functional classes for further study. The use of biologically relevant promoter classification will be vital for the identification of important regulatory signals and mechanisms. Such an approach should eliminate much of the noise that has resulted from indiscriminate mixing of different functional classes in genome-wide analyses, thereby conferring a clear advantage to computational studies.

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Competing interests statement
The authors declare no competing financial interests.

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