COMMENTARY

The eukaryotic transcriptional machinery: complexities and mechanisms unforeseen

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The temporal and spatial expression of specific genes is central to processes such as development, differentiation and homeostasis in eukaryotes, and is regulated primarily at the level of transcription. An understanding of the molecular basis for this regulation has presented a major challenge for the past 40 years. After early insights into genetic control mechanisms in prokaryotes, elegantly elaborated in the classic 1961 paper of Jacob and Monod, it was anticipated by many that similar principles would apply in eukaryotes. However, early studies in animal cells faced the problem of genomic complexity (including reiterated DNA sequences) and the lack of tractable genetic approaches. By the time I entered graduate school in the mid 1960s, the general RNA classes—ribosomal (rRNA), transfer (tRNA), messenger (mRNA) and the enigmatic heterogeneous nuclear (hnRNA)—had been recognized, and variations in the levels of these RNA classes were being defined during various growth, developmental, hormonal and viral responses. However, except for the early work from the laboratories of Max Birnstiel and Don Brown on the purification and structural analysis of the amplified rRNA genes of *Xenopus laevis*, essentially nothing was known about gene structure, transcriptional regulatory mechanisms or the basic transcriptional machinery in animal cells. Thus, the field was ripe for discovery of even the most fundamental principles of eukaryotic transcription, and a major question was how similar they would be to those of prokaryotes.

My interest in transcription was stimulated, as a Wabash College undergraduate in 1963, by a biochemistry course that covered recent work in bacterial genetic regulatory mechanisms. This interest was further heightened by a course taught by Sol Spiegelman in 1964, my first year of graduate studies in biochemistry at the University of Illinois. Although research opportunities in eukaryotic transcription were limited, I was able to join the laboratory of Bill Rutter just as he was moving to the University of Washington. Though not yet studying transcription *per se*, Rutter had begun a biochemical analysis of cell-specific protein synthesis during pancreatic development and had visions of extending this to the regulation of RNA synthesis. But because the pancreas was not yet amenable to transcription studies, I was forced to explore other model systems.

Nuclear RNA polymerases: multiplicity, structure and general functions

My early graduate work at the University of Washington was spent analyzing RNA synthesis by endogenous RNA polymerase in nuclei isolated from rat liver during hormonal responses and from developing sea urchin embryos. But although these studies allowed quantitation of RNA synthesis, they were not especially satisfying and provided little insight into transcription mechanisms or their regulation. I therefore opted to exploit the transcriptional machinery as an entry point into this area, with the ultimate goal of reconstructing specific transcription events using purified components. At that time, and after Sam Weiss' original description in 1959 of RNA polymerase activity in a crude nucleoprotein preparation from rat liver nuclei, the abundant (and soluble) bacterial RNA polymerase was being actively studied, and trace amounts of soluble activity from mammalian nuclei also had been reported. Studies by Widnell and Tata of the effects of salt and divalent metal ions on the base composition of RNA synthesized in isolated nuclei also raised the intriguing possibility of two different enzyme activities, but did not exclude template-related effects[1].

After realizing from initial studies that most of the mammalian RNA polymerase was tightly bound to chromatin as a result of its engagement in active transcription, I first had to develop new methods to quantitatively solubilize the RNA polymerase and remove the interfering DNA and histone components. A number of trials and some insight from a 1942 paper by Mirsky and Pollister on nucleohistone properties resulted in a relatively simple procedure: nuclear disruption and histone dissociation from DNA at high salt concentration, followed by DNA breakage and dissociation of RNA polymerase by sonication, and finally selective precipitation of DNA-histone complexes by rapid dilution to a low salt concentration. The resulting soluble enzyme preparation then was amenable to conventional purification. Remarkably, the initial chromatographic step on DEAE-Sephadex in February 1969 yielded three peaks of activity (designated RNA polymerases I, II and III) that showed template-dependent incorporation of all four ribonucleoside triphosphates, distinct salt and metal ion responses and reproducibly distinct chromatographic behavior[2] (**Fig. 1**). Seeing these three peaks of activity in the middle of the night on the initial run was one of the truly exhilarating "Eureka!" moments in my scientific career, and I slept little in the ensuing weeks as I further characterized the enzymes.

I first discovered RNA polymerases I, II and III in nuclei from sea urchin embryos—a fortunate choice because of the relative abundance of all three RNA polymerases in that organism, and because it afforded me opportunities for rare excursions from the laboratory (**Fig. 2**). I subsequently found similar activities in rat liver nuclei and in yeast.

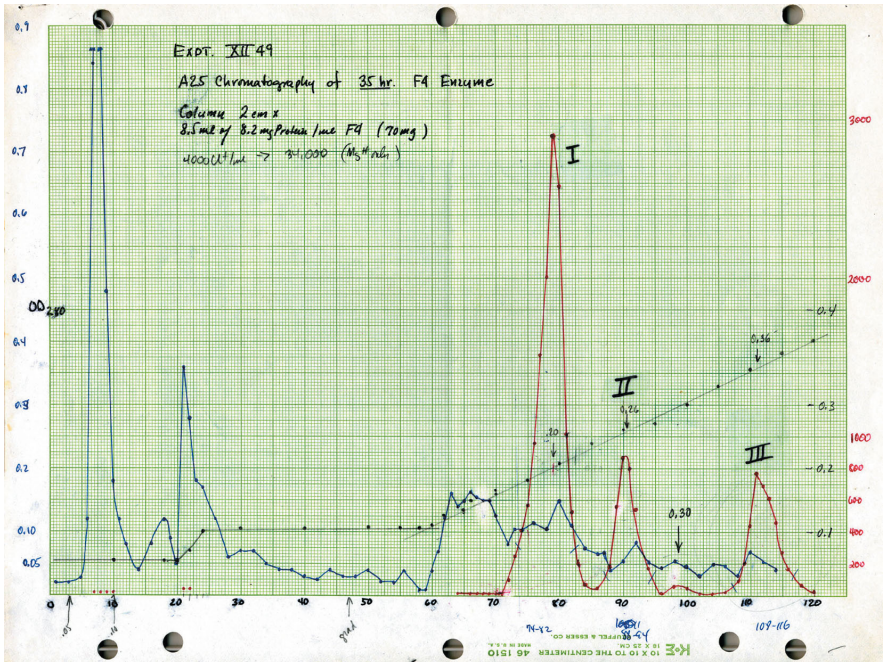

Figure 1 Chromatographic resolution on Sephadex A25 of the three nuclear RNA polymerases from sea urchin embryos. Original graph from chromatographic analysis (18 February 1969). Red, RNA polymerase activity; blue, total protein; black, ammonium sulfate gradient. Pol II activity is underrepresented because of suboptimal (low-Mg^{2+}) assay conditions.

(Disappointingly, none of the RNA synthesis–deficient yeast mutants of Lee Hartwell showed discernable defects in any of the solubilized RNA polymerases!) I also found that Pol I was localized within nucleoli, the sites of rRNA gene transcription, whereas Pol II and Pol III were restricted to the nucleoplasm[3]. This further suggested specific roles for each of the enzymes in the transcription of distinct classes of genes. With some trepidation, both for prematurely releasing my results and for giving my first public scientific presentation, I reported these findings at the April 1969 meeting of the Federation of American Societies for Experimental Biology. Needless to say, my findings were received with some acclaim, especially as a symposium speaker at the same meeting had reviewed current work and concluded that animal cells most likely contained a single enzyme! Coincidentally, my discovery of the three RNA polymerases occurred within weeks of publication of the landmark Burgess, Travers, Dunn and Bautz paper describing bacterial sigma factor and its effect on selective transcription by *Escherichia coli* RNA polymerase[4], further enhancing my excitement over future studies.

During a subsequent postdoctoral stint at the Carnegie Institution of Washington with Donald Brown, a pioneer in studies of gene structure and function in animal cells, Ron Reeder and I attempted to show that purified RNA polymerase I could accurately transcribe purified rRNA genes. To my great surprise and disappointment, this could not be demonstrated and I began to wonder about the possibility of other essential factors or even the necessity of a chromatin context for specific transcription. Despite the failure of this key experiment, I learned from Brown about the advantages of *Xenopus* as a model system and the necessity for good assays to ask specific questions about gene control.

In my subsequent position in the department of biological chemistry at Washington University School of Medicine, and as a prelude to further functional studies with purified genes and enzymes, I set out with eager young colleagues to purify all three enzymes to homogeneity and to rigorously establish their specific functions. In the latter case, Roberto Weinmann showed that purified RNA polymerases I, II and III could be distinguished on the basis of differential sensitivities to the mushroom toxin α-amanitin. By monitoring the α-amanitin sensitivities of specific transcription events by endogenous (engaged) RNA polymerases in isolated nuclei, he documented the synthesis of rRNA by Pol I, the synthesis of adenovirus pre-mRNA by Pol II and the synthesis of cellular 5S and tRNA and adenovirus VA RNA by Pol III[5,6]. These results strengthened the case for global regulation of the major classes of RNA through distinct enzymes and, perhaps more importantly, gave us important insights into model gene systems to reconstitute specific transcription events with specific RNA polymerases.

Meanwhile, to ascertain the structural

Figure 2 Robert Roeder collecting sea urchins in the frigid waters of the Strait of Juan de Fuca (1968).

basis for the functional differences between the RNA polymerases, Lawrence Schwartz and Virgil Sklar rigorously purified substantial amounts of all three enzymes from mouse plasmacytomas. Comparative analysis clearly showed complex and distinct subunit structures for all three enzymes, although there seemed to be at least two common subunits[7]. These results confirmed and extended preliminary studies from the Chambon and Rutter labs on Pol I and Pol II. Remarkably, the phylogenetically conserved eukaryotic enzymes are now known to contain 14 (Pol I), 12 (Pol II) and 17 (Pol III) subunits (the products of 31 distinct genes), including 5 shared subunits and enzyme-specific homologs to the β-, β'- and α-subunits of *E. coli* RNA polymerase. Clearly, the different mammalian enzymes were not derived from subtle modifications of a single enzyme, as would prove to be the case for the bacterial enzyme, which acquires different initiation specificities with distinct sigma factors. Thus, the studies of the mammalian enzymes provided the first indication, in eukaryotes or prokaryotes, of transcriptional regulatory mechanisms involving gene-specific structural variations in the general transcription machinery, as well as early indications of levels of control and complexity far beyond those existing in prokaryotes.

Reconstitution of accurate transcription: general initiation factors and mechanisms

With purified enzymes in hand and their general functions established, the next challenge was to achieve specific initiation. Focusing on the small and highly reiterated 5S RNA genes, which had been isolated by Don Brown and shown to be actively transcribed in oocytes, Carl Parker showed in 1976 that purified Pol III, but not Pol I, Pol II or *E. coli* RNA polymerase, could accurately transcribe 5S RNA genes in purified chromatin from immature oocytes but not in total cellular or cloned 5S DNA templates[8]. This long-awaited result—the first demonstration of accurate transcription by a eukaryotic RNA polymerase in a reconstituted cell-free system—greatly excited us and was significant in several respects. It suggested the presence of essential (chromatin-bound) RNA polymerase–specific accessory factors, cast doubt on the significance of the very popular studies elsewhere of chromatin transcription by bacterial RNA polymerases, validated our biochemical approach and stimulated studies with purified DNA templates. By 1978 we were able to show accurate transcription of cloned 5S RNA, tRNA and adenovirus VA RNA genes by purified Pol III in conjunction with soluble subcellular fractions from *Xenopus* oocytes and human HeLa cells[9,10]. Shortly thereafter, and with new information from the laboratory of Jim Darnell on the location of transcription start sites for pre-mRNA synthesis in the adenovirus genome, Tony Weil was able to show accurate transcription initiation (at the natural start site) on an adenovirus DNA fragment by purified Pol II and a crude subcellular fraction[11]. Don Luse also showed accurate transcription initiation from the β-globin promoter using the same assay. These very exciting findings immediately prompted a biochemical analysis of general and gene-specific transcription initiation factors for a variety of genes, and provided assays for determining DNA sequence requirements for transcription initiation. However, the permissive transcription in HeLa cell extracts of promoters that are naturally active only in adenovirus-infected or erythropoietic cells led us to believe that we were dealing with general initiation factors and left unexplained the basis for cell-restricted transcription.

Although a multitude of labs focused on the determination of DNA sequence requirements for initiation, using cell-free transcription and transfection assays, we felt that the unknown protein factors were the most important (and challenging) part of the problem and returned to the cold room to fractionate HeLa extracts. In 1980, Jacki Segall, Takashi Matsui and Tony Weil were able to distinguish two factors, TFIIIC and TFIIIB, that are generally required for transcription of class III genes and several factors, including TFIID, that were required for transcription of class II genes[12,13]. In early 1983 my laboratory moved to The Rockefeller University, where we continued the purification of general initiation factors. Work from my own and other laboratories over the next decade or more established that: (i) the two Pol III factors contain at least nine distinct polypeptides; (ii) there are six Pol II factors (TFIID, TFIIA, TFIIB, TFIIE, TFIIF and TFIIH) with a total of about 32 distinct polypeptides; (iii) there are several Pol I factors; (iv) the Pol I, Pol II and Pol III factors are structurally and functionally distinct; and (v) the general initiation factors are highly conserved from yeast to humans, thus paving the way for genetic analyses of the factors in yeast. The structural complexity of the individual, enzyme-specific classes of initiation factors was quite surprising, especially in light of their common usage by most or all genes within a given class, and the earlier established complexity of the cognate RNA polymerases. However, these accessory factors clearly offered the possibility for yet another layer of gene control through variations in the their levels or activities or through selective interactions with gene-specific regulatory factors.

In an attempt to make sense of this complexity, and anticipating that specific DNA-protein and protein-protein interac-

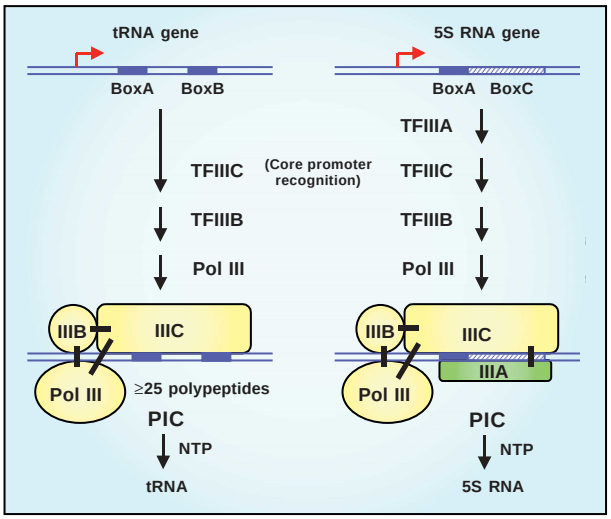

Figure 3 General initiation factors and PIC assembly pathways for class III genes with internal promoter elements, and activation by a gene-specific factor. On the tRNA gene promoter, assembly of a functional PIC containing Pol III and general initiation factors (yellow) is nucleated by stable binding of TFIIIC to the BoxA and BoxB elements. In contrast, assembly of a PIC on the divergent promoter of the 5S RNA gene is more highly regulated and requires prior binding to BoxA and BoxC of the 5S gene–specific activator TFIIIA, which interacts with and stabilizes TFIIIC binding. For both genes, TFIIIB is recruited by TFIIIC and Pol III is recruited by TFIIIB and TFIIIC. Solid black bars indicate interactions between Pol III and the various factors. NTP, ribonucleoside triphosphates. Adapted from ref. 23.

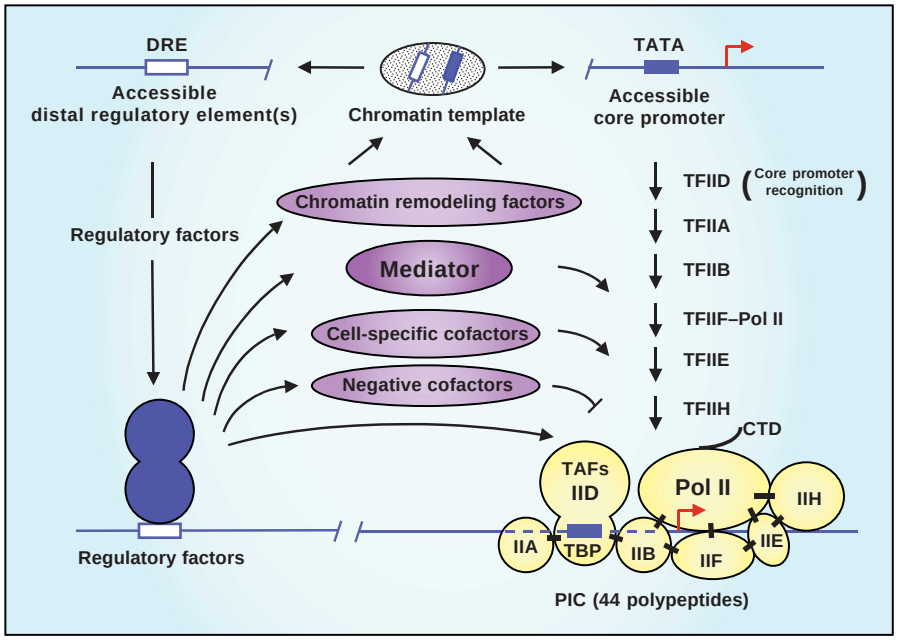

Figure 4 General initiation factors and PIC assembly pathway for class II genes with a TATA-containing core promoter, and regulation by gene-specific factors and interacting cofactors. Assembly of a PIC containing Pol II and general initiation factors (yellow) is nucleated by binding of TFIID to the TATA element of the core promoter. A model for the regulation of PIC assembly and function involves, sequentially: (i) binding of regulatory factors to distal control elements; (ii) regulatory factor interactions with cofactors that modify chromatin structure to facilitate additional factor interactions; and (iii) regulatory factor interactions with cofactors that act after chromatin remodeling to facilitate, through direct interactions, recruitment or function of the general transcription machinery. TAFs, TBP-associated factors. Adapted from ref. 23.

tions would serve as points of control by other factors, we undertook mechanistic studies with purified RNA polymerases and partially purified initiation factors. Studies by Andrew Lassar, Jim Bieker, Noboru Nakajima, Mike Van Dyke and Michele Sawadogo led to the definition of core promoter recognition factors and pathways for the ordered assembly of functional preinitiation complexes (PICs)[14–16] for class III genes (tRNA, VA RNA) containing the most common class III promoter elements (A and B boxes) and for class II genes containing the most common class II core promoter element (TATA box), as shown in **Figures 3** (left) and **4** (right). These pathways emphasize primary core promoter recognition by TFIIIC and by TFIID, respectively, forming complexes that nucleate the assembly of the remaining cognate factors and RNA polymerases into the PIC.

Beyond our initial studies in the mid 1980s, many laboratories have contributed to the elaboration of these pathways. Of special note are contributions of the laboratories of Phil Sharp and Lenny Guarente[17], as well as Danny Reinberg, to elucidation of the later steps in the Pol II pathway[18]. A yeast TATA-binding polypeptide (TBP) that could substitute for human TFIIID in PIC assembly and function was described in 1988 by Sharp and Guarente and by Chambon and Sentenac, and proved crucial not only for the more detailed mechanistic studies, but also for the isolation and characterization by several groups of metazoan TBPs and TBP-associated factors within the >1 MDa TFIID complexes. Masami Horikoshi and Alex Hoffmann spearheaded our efforts on this front, proceeding necessarily through an ortholog walk (based on conserved sequences) from *Saccharomyces cerevisiae* TBP, through *Schizosaccharomyces pombe* TBP, to human TBP and associated factors. X-ray structural analyses of TBP and TBP-TATA and higher-order complexes by the laboratories of Stephen Burley and Paul Sigler also provided important insights into early steps in PIC assembly[19]. More recently, the remarkable work of Roger Kornberg on the X-ray structure of RNA polymerase II has given us further insights and hope for a structural analysis of the complete PIC[20]. Given that RNA polymerases and general initiation factors are the ultimate targets of regulatory factors, these basic assembly pathways revealed many potential points of contact for receiving signals from interacting regulatory factors or cofactors.

Regulation of transcription: sequence-specific factors

By 1971, genetic and biochemical studies had firmly established transcriptional regulation by sequence-specific DNA-binding proteins (activators and repressors) in prokaryotes, and later mechanistic studies showed direct interactions with RNA polymerase. In the absence of documented cell- and promoter-specific binding factors, the permissiveness of our cell-free systems and the observations by others of short-lived nuclear RNAs, post-transcriptional RNA processing pathways and global chromatin activation domains left me wondering about the possibility of somewhat divergent regulatory mechanisms in eukaryotes. Nevertheless, the notion of regulation by gene- and cell-specific transcription factors remained most appealing to me, and I believed that in the absence of tractable genetic approaches in animal cells, our reconstituted cell-free systems provided a necessary and powerful approach to this problem. In 1979 (ref. 21), this approach led to David Engelke's identification and purification of the 5S gene–specific activator TFIIIA, and to our demonstration of site-specific binding to the internal 5S promoter that had just been mapped by Don Brown. The primary sequence of TFIIIA was revealed through cognate cDNA cloning by Ann Ginsberg in 1984 (ref. 22).

TFIIIA was the first of many sequence-specific DNA-binding transcription factors to be identified, purified and cloned in eukaryotes, and holds the additional distinction of being the prototype zinc finger protein. This structure, first proposed by Aaron Klug, is the predominant DNA-binding motif in eukaryotic regulatory factors. Mechanistic studies also showed that TFIIIA binding to the 5S promoter precedes and facilitates the sequential binding of TFIIIC, TFIIIB and PolIII[14] (**Fig. 3**, right). These studies with TFIIIA were the first to show a primary role for a gene-specific regulatory factor in RNA polymerase function in eukaryotes. However, in distinct contrast to the bacterial mechanism involving direct RNA polymerase–regulatory factor interactions, the effect on eukaryotic RNA polymerase is mediated indirectly through a host of other initiation factors. This more complicated mechanism, which allows for more subtle regulation through multiple inputs, has proved to be a dominant theme in eukaryotes.

Our success with TFIIIA gave me more confidence than ever about the validity of my views on gene- and cell-specific transcription factors and led my group and others to search, using a similar functional approach, for such factors for class II genes. The earliest factors that were so identified and shown to act through promoter-specific elements were Sp1 (discovered by Bob Tjian in 1983), HSF (by Carl Parker in 1984) and USF (by us and others in 1985). Along with earlier studies of the glucocorticoid receptor by Jan-Åke Gustafsson and Keith Yamamoto in 1981 and later studies of genetically identified yeast regulatory factors in 1985, these studies provided compelling evidence for factors that regulate RNA polymerase function by binding to distal DNA control elements. In keeping with the paradigm established for TFIIIA, the earliest *in vitro* mechanistic studies implicated TFIID, the primary core promoter-recognition factor, as a presumptive activator target[15] and stimulated intense efforts to further characterize this factor. At present, dozens (perhaps hundreds) of sequence-specific DNA-binding factors, both ubiquitous and cell specific, have been identified and implicated in various genetic regulatory pathways.

Yet another layer of complexity: diverse transcriptional coactivators

Because regulated expression of the diverse protein-coding genes in the nucleus underlies essentially all aspects of physiology, studies of transcriptional regulation became increasingly focused on Pol II–related regulatory mechanisms. By the early 1990s, the availability of cell-free systems reconstituted with highly purified preparations of Pol II and general initiation factors made it possible to analyze an increasingly broad spectrum of regulatory factor functions in defined systems. Given the specificity intrinsic to the DNA-binding regulatory factors, the structural complexity of the general transcription machinery (their ultimate target) and the documented interactions between regulatory factors and several general initiation factors, it was thought that the essential components were in hand and that detailed regulatory factor mechanisms could be elucidated. Quite surprisingly, however, sequence-specific DNA-binding activators were unable to function in systems reconstituted with these components and we were forced, once again, to postulate and search for other factors. Further biochemical studies led to the discovery in several laboratories of various coactivators that were essential for activator function[23]. The most significant of these, the so-called 'Mediator', is a ~25-subunit complex that has proven to be the main conduit for communication between DNA-bound activators and the general transcriptional machinery (**Fig. 4**). Mediator activities were first discovered in 1991 in extracts from yeast by Roger Kornberg[24] and in extracts from HeLa cells by Michael Meisterernst in my lab[25]. The yeast Mediator was first purified by the Kornberg lab in 1994 (ref. 26), and the human Mediator was first purified by Joe Fondell in my lab in 1996, as the thyroid hormone receptor–associated protein complex[27]. Earlier studies by Rick Young had identified essential yeast Mediator components, SRBs, that interact genetically with Pol II and reside in a Pol II holoenzyme[28], presaging the soon-to-be-described mechanisms involving direct Mediator interactions with Pol II. To date, a number of site-specific DNA-binding regulatory factors have been shown to function through direct interactions with distinct Mediator subunits, and the huge complex is viewed as a multivalent control panel that integrates diverse signals from both activators and repressors[29].

Several more highly regulated and highly selective coactivators are now known to exist. Two that were first identified by Yan Luo in my laboratory through biochemical complementation assays include the B-cell-specific OCT coactivator (OCA)-B (ref. 30) and, most recently, the S-phase-specific OCA-S (ref. 31). These factors interact with the ubiquitous DNA-bound OCT-1 on immunoglobulin and histone-2B promoters, respectively, and provide a remarkable example of selective cell-specific promoter activation through distinct coactivators that act, in part, through a common DNA binding factor. OCA-B is notable because its discovery in 1992 provided a new paradigm for tissue-specific gene regulation, and OCA-S is notable because it contains a nuclear form of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase as a key component and is regulated by NAD^+ and NADH. Emphasizing the continuing importance of the biochemical complementation assays that we developed, it seems likely that OCA-S, because of the roles of its component parts in other essential cellular pathways, would not easily have been identified by other means such as genetics.

Studies in many other laboratories have identified another important group of cofactors—coactivators and corepressors—

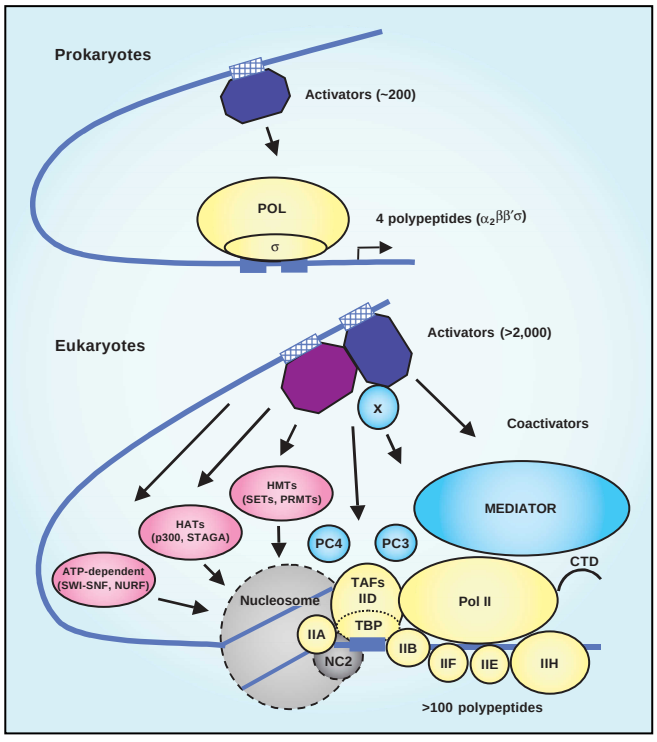

Figure 5 Comparison of transcription factors and activation mechanisms in prokaryotes and eukaryotes. In each case, the ultimate purpose of DNA-binding activators is to mediate RNA polymerase recruitment and function. But whereas activators interact directly with RNA polymerase subunits (primarily α and σ) in prokaryotes, the effects of activators on RNA polymerase II in eukaryotes are mostly indirect and involve interactions with diverse cofactors that modify chromatin structure and facilitate recruitment of RNA polymerase II and general initiation factors. Chromatin remodeling factors (pink) include ATP-dependent factors, histone acetyltransferases (HATs) and histone methyltransferases (HMTs), examples of which are indicated in parentheses. Cofactors (blue) that act at the level of DNA include the Mediator, general positive cofactors such as PC3 and PC4, cell-specific factors (X) such as OCA-B and OCA-S, and negative cofactors such as the TBP-interacting NC2. CTD, C-terminal domain of large subunit of Pol II. For further details see text and ref. 23.

that act through modifications of chromatin structure. These include ATP-dependent factors that alter the structure or position of nucleosomes, and factors that effect covalent modifications of the histone tails. The resulting chromatin alterations are thought to facilitate or prevent PIC formation or proper function. This suggests the sequential use of a complex (and potentially variable) set of cofactors in the activation of any given gene by one or more (usually a group of) DNA-bound regulatory factors (**Fig. 4**).

Conclusions and perspectives

Since the discovery of the three nuclear RNA polymerases over 34 years ago, we have come to appreciate, largely through biochemical approaches, the remarkable complexity of the general transcriptional machinery and the increasingly large set of cofactors needed for regulatory factor function. This complexity far exceeds that observed for the prokaryotic transcriptional machinery (**Fig. 5**) and was not even remotely anticipated three or four decades ago. Why all this complexity? We now know that the human genome contains some 35,000 genetic loci, many with multiple initiation and splice sites, and (based on sequence motifs) that more than 2,000 of these loci are likely to encode DNA-binding regulatory factors. We also know that gene expression must be exquisitely regulated and finely tuned during development, differentiation and homeostatic responses. To this end, cells seem to have evolved not only the expected highly complex array of DNA-binding regulatory factors, but also a complex machinery through which these regulatory factors act, thus affording a galaxy of possible outcomes from a limited number of signaling pathways. More specifically, this complex machinery allows: (i) global control mechanisms for the major classes of RNA; (ii) mechanisms to activate or repress genes packaged within natural chromatin structures; (iii) multiple targets for the cooperative functions of combinations of DNA-bound regulatory factors; (iv) mechanisms for the integration of multiple signaling pathways; and (v) a basis for alternative (redundant) gene activation and repression pathways.

Major challenges still lie ahead. One will be to integrate the functions of the relevant activators and coactivators and the general transcription machinery on natural chromatin templates in systems reconstituted with completely defined components, and to detail, for individual genes, the mechanisms involved. These studies can be complemented and, in part, guided by various genomic, proteomic and genetic studies; they should ultimately be directed toward an understanding of the function of complex and far-distal enhancers, as well as the apparent coupling of various events of transcription (initiation, elongation and termination) and RNA processing and transport. Another challenge will be to delineate the structure and dynamics of the PIC and its interacting activator-cofactor complexes. Finally, there is hope that while learning how the cell uses its transcription machinery for normal gene regulation, we might also deduce ways to selectively regulate and control aberrant gene expression.

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