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Mediator special issue

Mediator and the mechanism of transcriptional activation

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Mediator was discovered because of its activity in a yeast RNA polymerase II (pol II) transcription system – it is needed for the system to respond to a transcriptional activator. Mediator is the central link in the enhancer→activator→Mediator→pol II→promoter pathway. The transduction of regulatory signals through this pathway is crucial for transcription of almost all pol II promoters in all eukaryote organisms.

Introduction

The ultimate goal of research on transcription is an understanding of transcriptional control. In the case of bacteria and bacteriophage, this goal has been largely achieved. Transcriptional repressor and activator proteins, which are responsive to environmental stimuli, bind to DNA sequences that are adjacent to promoters and exert effects directly on RNA polymerase. Repressors prevent polymerase binding to the promoter, whereas activators contact polymerase and increase its affinity for the promoter or stimulate the transition from a closed to an open polymerase–promoter complex (formation of a 'transcription bubble', in which the DNA double helix is melted to facilitate the initiation of transcription).

At one level, a similar basis was found for control of transcription in eukaryotes. A signal of intracellular or environmental origin affects the state of a regulatory protein – its nuclear localization, its half-life, or its activity – with a consequent effect on transcription. At another level, the problem remained: how are multiple regulatory signals, which impinge on complex eukaryotic promoters, processed and transmitted to RNA polymerase II (pol II)? The solution of this problem has been found in features of the transcription machinery that are unique to eukaryotic cells.

The central components of the transcription machinery are the same in bacteria and eukaryotic cells. The RNA polymerases share a conserved core and common transcription mechanism. The initiation factors – σ in bacteria and a set of general transcription factors (GTFs) in eukaryotes – are more distantly related, but function in a similar manner in promoter recognition, promoter melting, abortive initiation and promoter escape. Where bacterial and eukaryotic systems truly diverge is in the targets of regulatory proteins. In contrast with the direct targeting of RNA polymerase in bacteria, there are intermediary factors in eukaryotes: chromatin and Mediator. Chromatin of eukaryotes, which is based on a histone octamer enveloped by DNA, and Mediator, a giant multiprotein complex, have no counterparts in bacteria. They represent a new layer interposed between the regulatory proteins and RNA polymerase. This layer must account for the greater complexity of regulation in eukaryotes and the consequent capacity for cell differentiation and development.

Discovery of Mediator

Although ostensibly an exercise of biochemistry, involving the fractionation of a yeast extract, the isolation of Mediator was anything but straightforward. The reason for this was to do with the complexity of the transcription system, the vagaries of the protein factors and the very definition of transcriptional activation. The yeast system was advantageous for the research: (i) it provided an early indication of the existence of Mediator and, thus, motivation for persisting despite the difficulties; and (ii) it provided validation of the final result, establishing both the physiological relevance and the broader implications of Mediator for control of transcription.

The earliest evidence for Mediator came from biochemical studies in yeast. It was previously shown that overexpression in yeast of one activator interferes with the activation of pol II transcription by another [1]. This effect, termed 'squelching', was attributed to competition between activators for a common target that was present in a limiting amount in yeast. The target was believed to be a component of the pol II transcription machinery – either one of the GTFs or pol II itself. This idea was proved by the demonstration of activator binding to the TATA-binding protein (TBP) subunit of the GTF TFIID [2]. Activators were subsequently shown to bind to TFIIB, TFIIH and pol II [3–5]. The promiscuity of activator interactions did not shake confidence in a direct mechanism of transcriptional activation. However, the widespread belief in a direct mechanism was finally challenged by analysis of squelching in a crude yeast pol II transcription system *in vitro* [6]. On the one hand, addition of an excess of any of the GTFs or of pol II failed to relieve squelching, which argued against these proteins being the activator target. On the other hand, addition of a crude protein fraction from yeast did relieve squelching, and the activity of this crude fraction was termed Mediator.

For isolation of Mediator from the crude yeast fraction, a better assay than the relief of squelching was required.

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Relief of inhibition is, in general, a poor basis for protein fractionation. It can lead to the isolation of molecules that sequester, degrade or otherwise antagonize the inhibitor, rather than to the physiologically relevant target. This difficulty was overcome with the advent of a reconstituted yeast pol II transcription system [7]. Although the components of the system (pol II and GTFs) were far from pure, they were sufficiently free of Mediator that transcriptional activation depended on the addition of Mediator, for which the same the crude fraction as previously used to relieve squelching was employed.

Even the initial reconstituted pol II transcription system did not provide a basis for the isolation of Mediator, as the requirement for Mediator in the system was variable. The components of the system were evidently variable, and the solution was to purify them completely and arrive at a fully defined, reconstituted system. This was a formidable task because of the limitations of the yeast system – the transcription signal is 1000-times smaller than that of the human HeLa system due to much lower concentrations of pol II and GTFs in yeast and a greater abundance of inhibitors in the starting extract. After several years, pol II and four of the five GTFs were purified to homogeneity from yeast, and the remaining GTF, TFIIH, was enriched by five chromatographic steps [8]. A sixth step was needed to obtain homogeneous TFIIH, but the activity was mysteriously lost in the last step. At the penultimate stage, with a nearly fully defined system, the response to transcriptional activators and requirement for Mediator were still variable. The significance of Mediator and even its existence were called into question.

The solution of the Mediator problem lay in the final purification of TFIIH. It emerged that several subunits of TFIIH, crucial for function, were lost during isolation. The occurrence of these subunits was not recognized because their levels were very low in the nearly pure protein. The traces that remained were required for transcriptional activity and were removed completely in the last step of isolation. When this issue was overcome and transcription was reconstituted with pure, fully functional TFIIH [9], the response to activators became reliable. The previous variability, and occasional activator response in the absence of added Mediator, had apparently arisen from contaminating Mediator in incompletely purified TFIIH.

With a consistent assay as a guide, Mediator was finally purified to homogeneity [10]. The purified protein had several notable characteristics. It was large and complex, comprising ~20 polypeptides, with a total mass of 1 million Daltons. It was isolated in two forms, alone and in an even larger complex with pol II. And not only was it required for the response to a transcriptional activator, but it also stimulated transcription tenfold in the absence of an activator.

Perhaps most remarkably, thirteen Mediator subunits were products of genes identified by previous screens in yeast for mutations affecting transcriptional regulation [11]. These were disparate screens, performed on different promoters in different laboratories at different times. With the isolation of Mediator, the products of the genetic screens were united in a common biochemical entity. This intersection of biochemical and genetic analysis in yeast was of particular significance for two reasons. First, it established the relevance of Mediator and of its functions assayed *in vitro* to transcriptional activation *in vivo*. Second, many of the genetic screens were for mutations affecting transcriptional repression rather than activation. Thus, it emerged that Mediator is important for negative and positive control of transcription. Mediator might be capable of integrating multiple signals and processing regulatory information at the complex promoters of higher organisms.

With the benefit of hindsight, the genetic screens that identified Mediator subunits are sometimes viewed as harbingers of the Mediator idea. On the contrary, there was no suggestion of an intermediary factor between transcriptional activators and pol II before the analysis of squelching *in vitro*. Even after the idea of Mediator was put forward, the relationship to the genetic studies was unappreciated. Srbs (suppressors of RNA polymerase B), the largest group of genetically identified proteins that would later be found in Mediator, were initially regarded as 'TBP-associated factors (TAFs)', components of TFIID rather than of Mediator [12].

Conservation of Mediator

For nearly a decade, the 'TAF hypothesis' was favored over Mediator as a basis for transcriptional regulation. TAFs are well conserved between yeast and higher cells, and evidence was presented for a role of TAFs in communication between activators and pol II. Mediator subunits, by contrast, were apparently conserved poorly, if at all. Sequence comparisons revealed only a handful of homologues of yeast Mediator subunits in higher cells. Nevertheless, the proposal that TAFs represented a universal link between activators and promoters was eventually disproved, and counterparts of yeast Mediator were identified in mammals, *Drosophila* and all other eukaryotes investigated (see reviews by Conaway *et al.*, Kim and Lis, and Malik and Roeder in this issue).

The close correspondence between yeast and mammalian Mediators was initially shown by structural studies. Rudimentary analysis by electron microscopy (averaging 66 micrographs of protein in negative stain in a single direction of view) was remarkably informative [13]. More sophisticated analysis (thousands of images of protein in the frozen, hydrated state, averaged and reconstructed in 3D) confirmed the initial findings, in particular the following [14,15] (see review by Chadick and Asturias in this issue):

- Mediator preparations contain many identical particles, with a compact shape and a size of ~200×400 Å. Although biochemical evidence had been indicative of such a particle, the complexity was so great that the particle was scarcely imagined. Therefore, the first direct observation of Mediator was important; it proved the existence of Mediator as a discrete entity and gave the Mediator idea reality.
- Yeast and mammalian Mediators appeared similar in size, shape and, at the low resolution of the analysis, similar in internal structural detail.
- Both yeast and mammalian Mediators unfolded to a

crescent conformation and partially surrounded pol II in the complex formed between them.

The striking similarities of yeast and mammalian Mediators, in both morphology and conformational behavior, made a compelling case for conservation at a time when the issue was in doubt. Conservation of Mediator was further established by genomic and proteomic analyses. Sequence comparisons between yeast and mammalian Mediator subunits initially disclosed only eight homologues, but cross-species comparisons from yeast to other lower eukaryotes to higher eukaryotes eventually revealed homologues of nearly all yeast Mediator subunits in human cells [16,17]. At the same time, Mediator complexes isolated from various mammalian sources initially seemed to contain distinct, but overlapping, sets of subunits. A multidimensional mass spectrometric approach, however, revealed essentially the same set of subunits in all mammalian Mediators investigated [18] (see review by Conaway *et al.*).

The pol II holoenzyme

Studies of the Srb proteins provided the earliest evidence for a complex of Mediator with pol II [12]. The complex contained many additional proteins, but it was not extensively purified, so the significance of these proteins was unclear. The proteins doubtless included most, if not all, Mediator subunits as shown by the subsequent isolation of a stoichiometric Mediator–pol II complex [10]. The occurrence of this complex provides the best evidence for a regulatory mechanism involving Mediator–pol II interaction.

Partially purified preparations of the Srb–pol II complex also included TFIIB, TFIIE, TFIIF, TFIIH and the SWI/SNF chromatin-remodeling complex. These and other findings led to the 'holoenzyme' hypothesis: the notion of a preformed complex comprising pol II, Mediator and diverse transcription factors that would be recruited to a promoter by activator–Mediator interaction. Referring to such a preformed transcription complex as the pol II holoenzyme has led to confusion because the complex containing only Mediator and pol II was also originally referred to as pol II holoenzyme. The term is used here only in reference to a preformed transcription complex.

Analyses of Mediator localization *in vivo* have ruled out the proposal that a preformed transcription complex plays an important part in transcriptional regulation. Different distributions of Mediator and pol II were revealed by indirect immunofluorescence in *Drosophila* polytene chromosomes [19]. Recruitment of Mediator was shown to precede that of pol II to yeast promoters by chromatin immunoprecipitation [20–22]. Moreover, Mediator was recruited without pol II to activator-binding sites lacking associated 'core promoter' elements (e.g. the TATA box and transcription start site) [23]. The demise of the holoenzyme hypothesis does not necessarily exclude a role for Mediator in the assembly of a transcription complex, but the process evidently occurs in a step-wise rather than concerted manner (see reviews by Kim and Lis, and Malik and Roeder).

Mediator as a GTF

Perhaps the most telling result from genetic studies of Mediator in yeast has come from the use of a temperature-sensitive mutant of Srb4 in genome-wide expression analysis [24,25]. More than 5000 genes showed nearly the same dependence on Srb4 as on the largest subunit of pol II. Two conclusions could be drawn. First, Mediator is essential for transcription; it might be regarded as a component of the pol II transcription machinery, comparable in importance to the GTFs and to pol II itself. Second, Mediator functions at all pol II promoters in yeast and, by inference, all pol II promoters in all eukaryotes.

The involvement of Mediator in transcriptional activation is connected with its more general role in transcription. The effect of an activator is expressed as the ratio of transcription in its presence ('activated transcription') to that in its absence ('basal transcription'). The importance of the denominator in this equation is often overlooked. As described, the main obstacle to the first isolation of Mediator and proof of its existence was the derivation of a true basal transcription system that was unresponsive to an activator. The level of basal transcription in such a system is notoriously variable. It is often deliberately diminished by the choice of reaction conditions so as to magnify the effect of a transcriptional activator.

Work done over the years to identify and purify pol II and the GTFs has sought to maximize basal transcription. Despite these efforts, it is striking how poorly basal transcription by pol II compares with that by other RNA polymerases. The template efficiency (number of transcripts produced per template) of a purified pol II system is on the order of 0.01, and even this low value might overestimate the actual number of templates engaged in transcription because multiple transcripts are generated from a single template by transcription reinitiation. Furthermore, the template efficiency of a purified pol II system is typically much less than that of crude extract, even though the concentrations of purified pol II and GTFs are much higher in the purified system and the level of inhibitors is far less. There is a tendency to regard the low activity of a purified pol II system as a deficiency, but it might instead represent a genuine, important characteristic of the system. The regulation of transcription would be most effective if the basal system were essentially inert. Pol II and the GTFs might assemble at a promoter but be unable to initiate transcription in the absence of a regulatory signal. Only in the presence of activator and Mediator would the basal system be triggered to transcribe.

The low activity of a purified pol II system might thus be viewed as a success of protein purification, reflecting the removal of stimulatory factors, rather than as a failure, due to the isolation of one or more components in an inactive state. The real deficiency of the purified system could lie in the occurrence of any transcription at all. The low level observed might either represent a natural feature, which necessitates repression for tight regulation *in vivo*, or an artifact of the system reconstituted *in vitro*.

Two observations support the idea of Mediator as the key to transcription by an otherwise inactive basal system: (i) as already mentioned, Mediator is required for transcription at any level by almost all pol II promoters

in vivo; (ii) Mediator stimulates transcription in a purified pol II system even in the absence of a transcriptional activator [10,26,27] (Y. Takagi, unpublished). The stimulation might arise from the embrace by Mediator of pol II (as described earlier) and perhaps of the entire preinitiation complex, including the GTFs and DNA (see review by Chadick and Asturias). This embrace might stabilize the preinitiation complex, promoting its formation or its maintenance for multiple rounds of transcription [28], or it might do something more, affecting the conformation of the complex and its activity in the initiation of transcription.

Transcriptional activation

Strong evidence for direct activator–Mediator interaction has come from studies in human systems. Thyroid hormone receptor (TR) was isolated from hormone-induced human cells as a complex with Mediator [29]. The Mediator subunits were identified as TR-associated proteins (TRAPs) before the existence of a human Mediator was appreciated. Similarly strong interactions of sterol-regulatory element-binding protein (SREBP) [30], vitamin D receptor [31] and adenoviral E1A protein [32] with human Mediator led to the independent isolation of human Mediator in other contexts. These studies leave no doubt as to the validity of Mediator as a primary activator target; they further identify points of contact within the Mediator complex (see reviews by Conaway *et al.*, Kim and Lis, and Malik and Roeder).

As mentioned, Mediator interacts extensively with pol II. A pathway of communication from activator to Mediator to pol II is thus defined, but the mechanism of communication in this pathway remains to be elucidated. How does the series of interactions stimulate the initiation of transcription? Although this question could not be studied directly as yet, several related questions have been investigated. For example, does activator binding cause unfolding of Mediator from the compact appearance of the isolated particle to the crescent shape seen in a complex with pol II? Unfolding seems to be a necessity for pol II interaction, but only small structural differences have been revealed by electron microscopy between Mediator in free and activator-bound states (see review by Chadick and Asturias).

A related question, or really set of questions, concerns 'activator-bypass' experiments, the holoenzyme hypothesis and the issue of recruitment as a mechanism of transcriptional activation. Activator-bypass refers to the fusion of the DNA-binding domain of an activator directly to a Mediator subunit, omitting the activation domain. Expression of such fusion proteins in yeast can result in high levels of transcription of reporter genes bearing the requisite DNA-binding sites. Such observations were originally attributed to the recruitment of pol II holoenzyme, but the finding of a multi-step mechanism of initiation-complex assembly disproved the holoenzyme hypothesis (see earlier). Mediator functions as a promoter through its interaction with activator in the first step, and pol II and the GTFs enter the complex subsequently. We can understand activator-bypass as simply increasing the efficiency of the first step. Recruitment could still have a role if Mediator were to attract pol II or enhance the formation of its complex with the GTFs at the promoter. There is, however, no compelling evidence for recruitment as a general basis for transcriptional activation at the present time, and alternatives, such as a conformational effect of Mediator on pol II or on the entire initiation complex must also be considered.

No less important than the role of Mediator in transcriptional activation, but less well understood, is its role in repression. A major limitation in its investigation is the lack of an *in vitro* system for repression. It is difficult to study repression *in vitro* because of the abundance of non-specific inhibitors of transcription. An important advance has nonetheless been made regarding the role of the Med12–Med13–CDK8–CycC (or Srb8–Srb9–Srb10–Srb11 according to the old nomenclature [17]) complex, which is required for repression of a subset of yeast genes. The Med12–Med13–CDK8–CycC complex associates with Mediator and prevents activation (see review by Björklund and Gustafsson in this issue). The basis for promoter specificity and the generality of this mechanism have just begun to be investigated.

Mediator might perform yet more roles in transcription. As mentioned, Mediator associates with yeast and *Drosophila* promoters almost immediately upon the induction of transcription, significantly in advance of pol II and the GTFs. It might be imagined that Mediator participates in all subsequent events, including the remodeling of promoter chromatin prior to assembly of the initial transcribing complex. It has been thought that Mediator remains at a promoter together with GTFs following the initial transcribing event, directing the reinitiation of transcription as well [28].

Concluding remarks

The challenge of understanding the Mediator mechanism begins with the still unsolved problem of the mechanism of transcription. The structures and functions of the 60 proteins of a complete transcription initiation complex, including Mediator, must be determined. Ultimately, the relationship of Mediator with all aspects of transcription, including chromatin remodeling, transcription elongation, and transcription re-initiation must be elucidated.

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