Genetic assays to define and characterize protein–protein interactions involved in gene regulation

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Abstract

Transcription can be regulated during initiation, elongation, and termination by an enormous variety of regulatory factors. A critical step in obtaining a mechanistic understanding of regulatory factor function is the determination of whether the regulatory factor exerts its effect through direct contact with the transcription machinery. Here I describe the application of a transcription activation-based bacterial two-hybrid assay that is useful for the identification and genetic dissection of protein–protein interactions involved in gene regulation. I provide examples of how this two-hybrid system can be adapted for the study of “global” regulatory factors, sequence-specific DNA-binding proteins, and interactions that occur between two subunits of RNA polymerase (RNAP). These assays facilitate the isolation and characterization of informative amino acid substitutions within both regulatory factors and RNAP. Furthermore, these assays often enable the study of substitutions in essential domains of RNAP that would be lethal in their natural context.

1. Introduction

1.1. Background

The transcription process can be divided into three distinct steps: initiation, elongation, and termination. Transcription regulation can occur during each of these steps through the action of a large variety of regulatory factors that mediate their effect on transcription through direct interaction with the transcription machinery. In bacteria, transcription is mediated by a single, multisubunit RNA polymerase (RNAP). The bacterial RNAP core enzyme (subunit composition σ2/β/β′/α) contains all of the catalytic machinery required for the synthesis of RNA from nucleotides. However, to initiate promoter-specific transcription, the RNAP core enzyme must associate with a σ factor to form the RNAP holoenzyme. High-resolution crystal structures of the bacterial core enzyme, holoenzyme and elongation complex have been determined [1–5]. These structures, coupled with biochemical analysis, have revealed distinct structural features of RNAP that play important roles during transcription and are likely targets of regulation. The structure of the RNAP core enzyme resembles a crab claw (Fig. 1). The large β and β′ subunits comprise the bulk of the ~380-kDa molecular mass of the core enzyme and encompass the enzyme's active-center cleft. The enzyme's active center, marked by a stably bound Mg²⁺ ion, lies at the base of the active-center cleft, which accommodates the DNA template and the ~9 basepair (bp) RNA–DNA hybrid that forms during transcription. The core enzyme contains two other distinct channels: the secondary channel, which is the presumed entryway for substrate NTPs, and the RNA exit channel, through which the nascent RNA transcript is extruded during elongation (Fig. 1).

A critical step in the analysis of transcription factor function is the determination of whether the regulatory factor exerts its effects on transcription through direct protein–protein contact with RNAP. However, determining whether a regulatory factor contacts RNAP and, if so, identifying the targeted surface of RNAP, can often prove difficult because RNAP is a large, multisubunit enzyme.

A transcription activation-based bacterial two-hybrid system [6–8] has been demonstrated to be an effective tool with which to study protein–protein interactions that facilitate gene regulation [9,10]. The system provides a facile way to obtain evidence of direct interaction between regulatory factors and sub-domains of RNAP (see Appendix Note #1). In addition, the two-hybrid assay facilitates the in vivo isolation and characterization of informative amino acid substitutions that alter protein–protein interactions between regulatory factors and RNAP. Furthermore, because the two-hybrid assay enables the genetic dissection of essential sub-domains of RNAP in the context of an otherwise inessential fusion protein, amino acid substitutions that are lethal in their natural context can be isolated and studied in vivo. Below I provide an overview of the principles underlying the transcription activation-based bacterial two-hybrid assay and I describe various applications that allow this two-hybrid assay to be used to dissect protein–protein interactions involved in gene regulation.
1.2. Principles underlying the transcription activation-based bacterial two-hybrid assay

The development of the transcription activation-based bacterial two-hybrid assay grew from pioneering work illustrating that transcription in *Escherichia coli* can be activated by any sufficiently strong contact between a DNA-bound protein and an interacting protein domain tethered to a subunit of RNAP [8]. Specifically, Dove et al., demonstrated that protein–protein contact between a protein domain fused to a subunit of RNAP and a partner domain fused to a DNA-binding protein could increase transcription from a test promoter bearing a recognition site for the DNA-binding protein in the upstream region [6,8]. This increase in transcription from the test promoter occurs as a consequence of cooperative binding. Specifically, the interaction between the protein domain tethered to RNAP and its partner domain that has been tethered to DNA (via a sequence-specific DNA-binding protein) stabilizes the binding of RNAP to the test promoter, resulting in an increase in transcription.

Two versions of the two-hybrid assay have been developed, one that relies upon fusions to the α subunit of RNAP and one that relies upon fusions to the ω subunit of RNAP [6–8,11] (see Appendix Note #2). Here I will focus on the version of the bacterial two-hybrid assay that utilizes fusions to the α subunit of RNAP (Fig. 2). In this case, the assay takes advantage of the domain structure of the α subunit. Specifically, α consists of two independently folded domains, the α N-terminal domain (αNTD; residues 8–235) and the α C-terminal domain (αCTD; residues 249–329), which are connected by a flexible linker domain [12–14]. The αNTD mediates formation of the α dimer, and serves as a scaffold for assembly of the core enzyme. The two α subunits occupy distinct locations within RNAP; thus, each αNTD makes distinct interactions with other core subunits [5]. The αCTD is a DNA-binding domain that interacts with upstream promoter DNA during transcription initiation and is the target for many regulators of transcription initiation [12–14]. However, because interactions between α and the other core subunits are mediated exclusively by the αNTD, the αCTD is inessential for assembly of the core enzyme. Therefore, fusion proteins in which the αCTD has been replaced by a heterologous protein domain can be stably incorporated into RNAP. Use of the two-hybrid assay to study protein–protein interactions involves the overexpression of such fusion proteins in *E. coli* cells. Although the αCTD is not required for formation of the core enzyme, the presence of the αCTD is essential for cell viability (likely because of the important role the αCTD plays for transcription initiation at the ribosomal RNA promoters [15]). For this reason, the two-hybrid assay is performed in *E. coli* cells carrying an intact copy of the α subunit (expressed

Fig. 1. Structure of the *Thermus aquaticus* RNAP core enzyme [5]. β is dark blue, β’ is light blue, ω is green, α is in orange, αII is in grey, active-center Mg²⁺ is in magenta. Shown are two views that highlight the locations of the active-center cleft, secondary channel, and RNA exit channel.

Fig. 2. Transcription activation-based bacterial two-hybrid assay. (A) Shown is the RNAP holoenzyme bound to a promoter. The transcription start site is indicated by the arrow. Regions 2 and 4 of σ (σ₂ and σ₄) are depicted in contact with the promoter –10 and –35 elements. The α subunit is depicted in a manner emphasizing its domain structure; the αNTD, which mediates interactions with the other core subunits is connected by a flexible linker to the αCTD. (B) Transcription-based bacterial two-hybrid assay. Contact between protein domain Y (fused to λCI) and protein domain X (fused to the αNTD and linker; in place of the λCTD) activates transcription from a test promoter bearing a binding site for X/CI (O,2) upstream of the core promoter elements. The test promoter is fused to a lacZ reporter gene allowing transcription to be monitored by performing β galactosidase assays. (C) Schematic illustrating the use of the two-hybrid assay. Compatible vectors directing the synthesis of the α-X fusion and the λCI-Y fusion are introduced into reporter strain cells carrying the test promoter-lacZ fusion on an F episome. Cells carry a chromosomal deletion of the lacZ gene.
from the chromosomal *rpoA* gene). Thus, cells expressing an α fusion protein likely contain a mixed population of RNAP molecules containing two, one or no copies of the α fusion protein.

Typically, the DNA-binding protein that is used in the two-hybrid assay is the bacteriophage λ CI protein (λ CI). λ CI is a dimeric protein that consists of two independently folded domains, the λ CI NTD and the λ CI CTD. The λ CI NTD is a DNA-binding domain while the λ CI CTD mediates formation of the λ CI dimer [16]. λ CI fusions are constructed by fusing protein domains to the λ CI CTD (via a short, three alanine residue linker attached to the end of the λ CI CTD).

The two-hybrid assay utilizes an artificial test promoter that contains a binding site for λ CI (the λ operator) in the upstream region (centered at −62 with respect to the start site of transcription; see Appendix Note #3). This test promoter is fused to a *lacZ* reporter gene and introduced in single copy on an F episome into a strain of *E. coli* that carries a chromosomal deletion of the *lacZ* gene (Fig. 2). Thus, transcription from the test promoter can be monitored by performing β galactosidase assays. To determine whether two proteins (or protein domains) interact, one of the proteins is fused to α and the other protein is fused to λ CI. The α fusion and the λ CI fusion are each placed under the control of an inducible promoter on separate, compatible vectors. These fusions are then introduced into reporter strain cells. If the two proteins interact, induction of the two fusion proteins will result in an increase in transcription from the test promoter (as measured by an increase in β galactosidase activity; see Appendix Note #4).

### 1.3. Considerations when using the bacterial two-hybrid assay to study proteins that contact RNAP

As described above, when using the bacterial two-hybrid assay to study protein–protein interactions, one protein domain is fused to the α subunit of RNAP and another protein domain is fused to λ CI. However, it is important to note that if the protein domain that is fused to λ CI can contact a surface of RNAP, then this λ CI fusion might activate transcription from the two-hybrid test promoter on its own. For example, fusion of the *E. coli* elongation factor NusA to λ CI results in a λ CI-NusA fusion protein capable of activating transcription when bound upstream of the test promoter (S.L. Dove personal communication). The observed activation is the result of contact between NusA and its target within RNAP, the αCTD. In contrast, by fusing NusA to the αNTD and the αCTD to λ CI the interaction between NusA and the αCTD can be detected and genetically dissected in an isolated setting (i.e., a situation where the RNAP sub-domain is removed from its natural context in the intact RNAP core enzyme). In particular, co-production of an α-NusA fusion along with a λ CI-αCTD fusion in reporter strain cells results in an increase in transcription from the two-hybrid test promoter (S.L. Dove personal communication).

It should be noted that fusion of an RNAP-interacting protein to λ CI does not always result in a fusion protein capable of activating transcription on its own from the test promoter. In particular, the surface of RNAP that is targeted by the fused regulatory factor may or may not be accessible in the context of promoter-bound RNAP holoenzyme. Although the ability to study regulatory factors using such a one-hybrid assay can potentially be useful, it is advisable to first attempt assays where the regulatory factor of interest is fused to the αNTD to avoid the potential complications that can result from fusing RNAP-interacting proteins to λ CI.

### 2. Description of method

Below I discuss several different examples that illustrate how the transcription activation-based two-hybrid assay can be used to study the regulation of gene expression. It is important to note that the two-hybrid assay can be adapted not only to study regulatory factors that contact RNAP, but also to characterize the functional roles of interactions between two subunits of RNAP.

#### 2.1. Use of the transcription activation-based bacterial two-hybrid assay to detect and study protein–protein interactions between regulatory factors and RNAP

Different strategies can be employed to study protein–protein interactions between the regulatory factor and RNAP depending upon how much is known about the regulatory factor of interest.

**2.1.1. Use of the two-hybrid assay to study regulatory factors for which prior information suggests a target**

In cases where prior information suggests a target surface for a particular regulatory factor, the two-hybrid assay can be used both to provide validation of the predicted target and to establish an assay that allows the genetic dissection of the interaction in an isolated setting. To illustrate this particular use of the two-hybrid assay, consider the *E. coli* regulatory factors GreB and NusG, which both affect gene expression by directly targeting RNAP. In the case of both GreB and NusG, prior evidence suggests what surfaces of RNAP they contact to mediate their effects on transcription.

**2.1.1.1. Use of the two-hybrid assay to study the interaction between GreB and RNAP**

GreB is a 17.5 kDa protein that can stimulate promoter escape and rescue stalled elongation complexes by directly contacting RNAP [17,18]. GreB acts as a “global” regulatory factor, exerting its effects on transcription in a manner that does not require sequence-specific interaction with DNA. Structural work indicates that GreB consists of a N-terminal antiparallel coiled coil and a C-terminal globular domain [19,20].

![Diagram](image_url)

**Fig. 3.** Use of the transcription activation-based two-hybrid assay to study protein–protein interactions between “global” regulatory factors and RNAP. (A) Schematic of assay used to test the interaction between a regulatory factor (fused to the αNTD) and a sub-domain of RNAP (fused to λ CI). (B) Use of the two-hybrid assay to detect the interaction between NusG and the β coiled coil and the interaction between GreB and the rim of the secondary channel. PW 102 O2−62 reporter strain cells were transformed with the indicated α fusion and the indicated λ CI fusion (the amino acid residues that were fused are listed in parenthesis). Bar graph shows the results of β galactosidase assays performed as described at an IPTG concentration of 100 μM.
high-resolution structures of RNAP and GreB onto the low-resolution structure of an RNAP/GreB complex reveals that the C-terminal globular domain of GreB makes extensive interactions with the outer rim of the secondary channel of RNAP [19]. Fig. 3 illustrates that the interaction between GreB and the outer rim of the secondary channel can be detected using the bacterial two-hybrid assay. To do this, full-length GreB was fused to the α subunit of RNAP and residues 649–704 of β', which form part of the rim of the secondary channel, were fused to α.C. The fusions were placed onto compatible vectors under the control of an IPTG-inducible promoter and introduced into reporter strain cells (carrying the two-hybrid test promoter- lacZ fusion). Induction of both fusion proteins increases transcription from the test promoter by a factor of ~6 (whereas induction of either fusion protein on its own does not increase transcription from the test promoter). These data indicate that the interaction between GreB and the RNAP secondary channel can be detected in the bacterial two-hybrid assay. Furthermore, this assay provides a convenient tool to genetically dissect the interaction between GreB and RNAP.

2.1.1.2. Use of the two-hybrid assay to study the interaction between NusG and RNAP. NusG is a highly conserved elongation factor involved in the regulation of transcription pausing and termination. Like GreB, NusG does not make sequence-specific contact with DNA, but rather acts as a "global" regulatory factor. Structural and biochemical characterization of a NusG homologue, RfaH, indicate that RfaH interacts with a coiled coil domain located in the β' subunit, the β' coiled coil [21,22]. The β' coiled coil is also the main binding site for σ factors. The finding that RfaH interacts with the β' coiled coil has led to the proposal that NusG also interacts with the β' coiled coil. Fig. 3 illustrates how use of the bacterial two-hybrid assay enables the detection of the protein–protein interaction between NusG and the β' coiled coil (residues 262–309 of E. coli β'). As with the assay described above that detects the interaction between GreB and the rim of the secondary channel, this assay can be used to genetically dissect the protein–protein interaction between NusG and RNAP.

2.1.2. Use of the two-hybrid assay to study regulatory factors for which no prior information exists regarding a likely target surface

An advisable strategy for studying regulatory factors for which no prior information exists regarding their target site is to take advantage of the high-resolution structures of bacterial RNAP to predict exposed folded sub-domains of the enzyme to use in the two-hybrid analysis (see Appendix Note #5). This is most important for the large β' and β subunits. Inspection of the high-resolution structure of Thermus aquaticus RNAP [5] suggests one can parse E. coli β into 10 sub-domains in this manner. Similar analysis of the E. coli β' subunit yields 18 sub-domains that cover the entire gene. (List of recommended E. coli β fusions to test: 1–151, 151–451, 450–530, 528–589, 587–656, 528–656, 665–798, 703–795, 829–930, 930–1059, 1137–1226, and 1246–1342 [note this list includes the two E. coli specific insertions]; list of recommended E. coli β' fusions to test: 25–104, 114–190, 140–235, 193–230, 249–328, 264–308, 370–416, 516–573, 576–634, 648–701, 735–790, 820–882, 944–1021, 1023–1128, 1137–1243, 1154–1212, 1261–1307, and 1308–1347.)

As discussed above (see Section 1.3), when attempting to identify what surface of RNAP is contacted by a regulatory factor it is generally advisable to first try experiments where the regulatory factor is fused to α and sub-domains of RNAP are fused to βC. Therefore, the desired first set of experiments would involve testing whether the regulatory factor of interest, when fused to α, activated transcription specifically in the presence of any sub-domain of RNAP fused to βC. For example, to attempt to identify a target region located in E. coli β or β' one would perform assays using the 30 fusions recommended above; the number of fusions to test would become larger if α, ω, and σ were included in the analysis.

If a protein–protein interaction between the regulatory factor being studied and a sub-domain of RNAP is identified from this analysis it is important to validate that this interaction is functionally relevant, i.e. occurs in the natural context. For this purpose the two-hybrid assay can be used to identify amino acid substitutions in the RNAP sub-domain or the regulatory factor that specifically disrupt (or enhance) the protein–protein interaction. Demonstrating that such substitutions have effects in an in vitro or in vivo assay that measures the activity of the regulatory factor provides a convincing validation that the interaction detected in the two-hybrid assay is required for regulatory factor function. In this case it is often preferable to use substitutions isolated in the targeted region of RNAP to validate interactions detected in the two-hybrid assay (because it is sometimes difficult to control for non-specific effects – e.g. effects on protein stability – of substitutions that are located in the regulatory factor). However, this applies only to situations where substitutions in the targeted region of RNAP can be tolerated either in vivo or in vitro in the context of otherwise wild-type RNAP. (Note that in order to identify substitutions that specifically affect the interaction between a given regulatory factor and a particular sub-domain of RNAP one would ideally want to be able to assay more than one interaction of the sub-domain of RNAP; see below, Section 2.2.)

2.1.3. Adaptation of the “two-hybrid” assay to study protein–protein interactions between DNA-bound regulatory factors and RNAP

To adapt the two-hybrid assay to study DNA-bound regulators of transcription requires the construction of a new artificial test promoter. The new test promoter carries the DNA-binding site for the regulatory factor of interest in the upstream region (in place of the λ operator present in the canonical test promoter). Using this new test promoter, one then determines whether co-production of the regulatory factor along with an α fusion protein (containing the sub-domain of RNAP to be tested) results in an increase in transcription from the test promoter. Thus, these assays take advantage of the fact that the protein being studied already can bind DNA and does not need to be fused to a DNA-binding protein in order to be "displayed" upstream of the artificial test promoter. (Therefore, in this derivative of the two-hybrid assay only one fusion protein is employed.)

In this section, I will first describe a general strategy for the study of protein–protein interactions between DNA-bound transcription factors and RNAP. I will then discuss a specialized example of this approach for the study of DNA-bound regulatory factors that interact with the σ subunit of RNAP.

2.1.3.1. Assays to study DNA-bound regulatory factors that contact RNAP. A test promoter is constructed that contains the binding site for the regulatory factor being studied in the upstream region of the promoter (in place of the binding site for λCl). The DNA-bound regulatory factor is then tested for interaction with sub-domains of RNAP that have been fused to the σNTD. (Note that this version of the assay cannot be used to study factors that contact the σCTD.)

An example of the utility of this assay for studying interactions between DNA-bound regulatory factors and sub-domains of RNAP is shown in Fig. 4. In this case, the interaction between a DNA-bound regulator of elongation, the bacteriophage 21 Q antiterminalizer protein (21Q) [23], and the flap domain of the RNAP β subunit (β flap) can be studied [24]. The test promoter contains the binding site for 21Q centered ~66 base pairs upstream of the transcription start site of the test promoter. When 21Q and an α-β flap fusion are co-produced, transcription from the test promoter increases as a result of protein–protein contact between DNA-bound 21Q and the β flap moiety of the α-β flap fusion [24].
I have found that the optimal upstream position of the DNA-binding site can vary from protein to protein. Therefore, it is advisable initially to construct a panel of test promoters each having the binding site for the regulatory factor centered at a different position upstream of the core promoter elements. In particular, for initial studies a panel of seven test promoters with the regulatory factor binding site centered at –72, –70, –68, –66, –64, –62, and –60 should be constructed (provided that the size of the regulatory factor binding site does not limit the potential positions where it can be placed).

2.1.3.2. Assays to study DNA-bound regulatory factors that contact $\sigma$. Bacteria typically contain a number of $\sigma$ factors, each specifying recognition of a distinct class of promoters [25,26]. The primary $\sigma$ factor in E. coli is $\sigma^{70}$, and the $\sigma^{70}$-containing holoenzyme is responsible for most transcription that occurs during the exponential phase of growth. In the context of the RNAP holoenzyme, $\sigma^{70}$ makes direct contact with two conserved promoter elements, the –10 and –35 elements (consensus sequences TATAAT and TTGACA, respectively). All primary $\sigma$ factors share four regions of conserved sequence, regions 1–4 [27,28]; regions 2 and 4 contain DNA-binding domains responsible for recognition of the promoter –10 element and –35 element, respectively. In addition, $\sigma^{70}$ region 4 is also targeted by many regulators of transcription initiation and by at least one regulatory factor that acts during transcription elongation [29].

In this section I discuss a specialized example of the use of a one-hybrid assay to study DNA-bound regulatory factors that contact $\sigma$ region 4. This assay takes advantage of a one-hybrid assay that enables the detection and genetic dissection of the interaction between $\sigma$ region 4 and the promoter –35 element. In this assay, illustrated in Fig. 5, the test promoter contains the binding site for $\sigma$ region 4, a consensus promoter –35 element, upstream of the core promoter elements. Overproduction of an $\alpha$-$\sigma$ region 4 fusion in cells carrying this test promoter increases transcription from the test promoter as a result of protein–DNA contact between the $\sigma$ region 4 moiety of the $\alpha$-$\sigma$ region 4 fusion and the ectopic “–35 element”. This assay has been employed to study interactions between the promoter –35 element and $\sigma^{70}$ (see Appendix Note #6) and as well as interactions between the –35 element and $\sigma^{38}$ (the stationary phase $\sigma$ factor in E. coli) [30–32]. In principle, this one hybrid assay is easily adaptable to study protein–DNA interactions between $\sigma$ factors from any organism and their cognate promoter elements. Furthermore, the use of an $\alpha$-$\sigma$ fusion allows one to study $\sigma$ in the context of an essential fusion protein, thus allowing the isolation and characterization of amino acid substitutions in $\sigma$ that would be difficult (or impossible) to isolate in the context of full-length $\sigma$ (see below, Section 2.2).

One important thing to note about this assay is that wild-type RNAP does not utilize the ectopic “–35 element” to initiate transcription. This is due to the fact that there is no appropriately positioned –10 element in the context of the test promoter.

To study DNA-bound regulatory factors that contact $\sigma$ the test promoter is designed such that the DNA-binding site for the regulatory factor of interest is placed immediately adjacent to the ectopic “–35 element” (Fig. 5). Co-production of the regulatory factor along with the $\alpha$-$\sigma$ region 4 fusion increases transcription (to a greater extent than that observed with the $\alpha$-$\sigma$ region 4 fusion alone). The increase in transcription from the test promoter is the result of protein–protein interaction between the DNA-bound regulatory factor and the $\sigma$ region 4 moiety of the $\alpha$-$\sigma$ region 4 fusion that stabilizes the binding of $\sigma$ region 4 to the ectopic “–35 element” and activates transcription of the test promoter.

This strategy has previously been employed to study the interaction between $\sigma^{70}$ region 4 and a DNA-bound regulator of transcription initiation (the $\lambda$Cl protein, which, in the context of the bacteriophage $\lambda$, activates transcription from promoter P$_{R\lambda}$ through contact with $\sigma^{70}$ region 4) [31]. Furthermore, this strategy has also been used to study the interaction between $\sigma^{70}$ region 4 and a DNA-bound regulator of transcription elongation (the bacteriophage $\lambda$ Q antiterminator protein) [33]. For these assays, it is critical that, in the context of the artificial test promoter, the

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**Fig. 4.** Use of the transcription activation-based two-hybrid assay to study protein–protein interactions between DNA-bound regulatory factors and RNAP. Shown is a schematic of the test promoter used to detect the interaction between a DNA-bound regulator of transcription (21Q) and the flap domain of the $\beta$ subunit ($\beta$ flap).

**Fig. 5.** Assays to detect interactions that the $\sigma$ subunit participates in. (A) Schematic of the test promoter used to detect the interaction between $\sigma$ region 4 and a promoter –35 element. This test promoter contains an ectopic “–35 element” upstream of the core promoter –10 and –35 elements. Interaction between the $\sigma$ region 4 moiety of the $\alpha$-$\sigma$ region 4 fusion protein and the ectopic “–35 element” activates transcription of the test promoter. (B) Schematic of the test promoter used to detect the interaction between a DNA-bound regulator of transcription and $\sigma$ region 4. The test promoter contains the binding site for the regulatory factor upstream of the ectopic “–35 element”. Interaction between the DNA-bound regulatory factor and the $\sigma$ region 4 moiety of the $\alpha$-$\sigma$ region 4 fusion protein stabilizes the binding of $\sigma$ region 4 to the ectopic “–35 element” and activates transcription of the test promoter.
spatial relationship between the regulatory factor binding site and the ectopic “−35 element” is exactly the same as the spatial relationship between these elements in the natural context (i.e., in the context of the regulatory factor’s target promoter).

For assays performed with either σ70 region 4 or σ58 region 4, the optimal positioning of the ectopic “−35 element” has been empirically determined to be between –43 and –48 (i.e., centered at –45.5; see Appendix Note #7). However, the optimal positioning of the binding sites for the regulatory factor and σ factor may vary depending upon what particular regulatory factor/σ factor pair is being tested. Therefore, it is recommended that a panel of test promoters, each containing these elements at different positions relative to the core promoter, be constructed when using this one-hybrid assay to study DNA-binding factors that contact the σ subunit.

2.2. Use of transcription activation-based bacterial two-hybrid assay to study protein–protein interactions between two subunits of RNAP

The ability to genetically dissect the protein–protein interaction between two subunits of RNAP in an isolated setting enables amino acid substitutions that specifically affect the particular inter-subunit interaction to be identified. These substitutions can then be used to determine the role that a particular inter-subunit interaction plays during the transcription cycle. To illustrate this, I will describe how the two-hybrid assay was used to detect and functionally characterize interactions that occur between the σ subunit and the RNAP core enzyme.

When σ associates with the core enzyme to form the holoenzyme, two domains of σ, regions 2 and 4, make important interactions with two sub-domains of RNAP core, the β′ coiled coil and the β flap, that facilitate promoter binding. In particular, holoenzyme formation depends critically on a high-affinity interaction between σ70 region 2 and the β′ coiled coil [34,35] (recall that the β′ coiled coil is also the target for the elongation factor NusG). The interaction between σ70 region 2 and the β′ coiled coil is required for σ70 to make functional contact with the promoter –10 element [36]. In addition, an interaction between σ70 region 4 and the β flap, while not required for holoenzyme formation, is required for sequence-specific interaction with the promoter –35 element [37].

The transcription activation-based two-hybrid assay has been used to study the interaction between σ70 region 2 and the β′ coiled coil [38] as well as the interaction between σ70 region 4 and the β flap [10]. Below, I describe how the two-hybrid assay was used to isolate amino acid substitutions in σ70 region 4 that specifically affect the interaction with the β flap. This example illustrates how assaying more than one interaction of a particular domain of interest can facilitate the isolation of amino acid substitutions that affect one interaction without affecting the other. Such substitutions are useful because they allow the researcher to rule out trivial explanations for the effects of these amino acid substitutions. (For example, the researcher can rule out the possibility that a particular amino acid substitution that abolishes one of the interactions does so by affecting the stability of the fusion protein.)

In the case of the interaction between σ70 region 4 and the β flap, region 4 of σ70 was fused to the αNTD, and the β flap was fused to λCI. Thus, the λCI–β flap fusion protein activates transcription from the two-hybrid test promoter specifically in the presence of the α–σ70 region 4 fusion. Random mutations were introduced into the gene fragment encoding the σ70 moiety of the α–σ70 region 4 fusion by PCR and mutations that abolished or enhanced the interaction between σ70 region 4 and the β flap were isolated by plating reporter strain cells carrying the mutated α–σ fusion and the wild-type λCI–β flap fusion on appropriate indicator media.

To identify substitutions in σ70 region 4 that specifically affected the interaction with the β flap, but did not alter the ability of σ70 region 4 to bind DNA, the one-hybrid assay that detects the ability of σ70 region 4 to bind a −35 element was employed. As described above (Section 2.1.3.2), in this assay, interaction between the region 4 moiety of the α–σ70 region 4 fusion and an ectopic “−35 element” positioned upstream of the core promoter elements activates transcription from the test promoter (see Fig. 5). Thus, substitutions that weakened or enhanced the interaction between σ70 region 4 and the β flap in the two-hybrid assay were tested for their effects on the interaction between the region 4 moiety of the α–σ70 region 4 fusion and the ectopic “−35 element” using the one-hybrid assay. In this manner, substitutions that disrupted or enhanced the interaction between the σ70 region 4 moiety of the fusion and the β flap, but did not affect the ability of the σ70 region 4 moiety to bind to a −35 element were isolated [10]. The introduction of these amino acid substitutions into full-length σ70 enabled the demonstration (using reconstituted wild-type and mutant holoenzymes in vitro) that the interaction between σ70 region 4 and the β flap plays functional roles not only during transcription initiation, but also during transcription elongation [10,39].

When introduced into full-length σ70 and over-produced at the non-permissive temperature in cells encoding a temperature sensitive σ70 mutant, substitutions that disrupted the interaction between σ70 region 4 and the β flap were found to be lethal [10]. Thus, these studies illustrate how the two-hybrid assay can be useful for isolating amino acid substitutions in essential domains of RNAP that are lethal in their natural context. It is also important to note that even though a particular amino acid substitution in RNAP may be lethal in E. coli cells, functional studies of such amino acid substitutions in the context of otherwise wild-type RNAP can be performed in vitro by using mutant enzymes reconstituted from individual subunits or isolated intact from cells using specialized co-expression systems [40].

2.3. Materials

Below is a list of materials that can be used to perform the two-hybrid assays discussed above.

2.3.1. Plasmids needed

(1) pBRαL:N [7,41]: used for making fusions to α.
(2) pACtC32 [7,41]: used for making fusions to λCI.
(3) pAC(tac) [33]: used for synthesis of full-length regulators that bind DNA.
(4) pFW11 placCons–35C [31]: used for making new test promoters.

2.3.2. Cells needed

(1) XL1 blue (Stratagene): used for cloning/plasmid manipulation.
(2) FW 102 O2–62 [42]: reporter strain for performing two-hybrid assays using λCI fusions.
(3) CSH 100 [43]: used for construction of reporter strains.
(4) FW 102 [43]: used for construction of reporter strains.

2.3.3. Reagents/chemicals needed

(1) Luria-Bertani (LB) agar plates: 10 g bacto tryptone, 5 g yeast extract, 10 g NaCl, 15 g bacto agar in 1 L of distilled water. Autoclave LB-agar to sterilize. Once poured, plates should be stored at 4°C.
2. LB: 10 g bacto tryptone, 5 g yeast extract, 10 g NaCl in 1 L of distilled water. Autoclave LB to sterilize and store at room temperature.

3. Antibiotic stock solutions: 100 mg/ml carbenicillin (or ampicillin) in 50% ethanol/50% water; 25 mg/ml chloramphenicol (in ethanol); 50 mg/ml kanamycin (in distilled water; filter sterilize); 100 mg/ml streptomycin (in distilled water; filter sterilize). These are 1000× stock solutions and should be stored at −20°C.

4. IPTG stock solution. To make a 100 mM stock concentration of isopropyl-β-D-thiogalactoside (IPTG) dissolve 0.238 g in 10 mL of distilled water, filter sterilize, aliquot, and store at −20°C.

5. X-gal stock solution. Make a 40 mg/ml stock of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in Dimethylformamide (DMF), store at −20°C.

6. Z-buffer: 16.1 g Na₂HPO₄·7H₂O, 5.5 g NaH₂PO₄·H₂O, 0.75 g KCl, 0.246 g MgSO₄·7H₂O, add distilled water to 1 L, adjust pH to 7.0 (if necessary). Do not autoclave. Immediately before using Z-buffer add β-mercaptoethanol (BME) to a final concentration of 0.27%.

7. 0.1% sodium dodecyl sulfate (SDS) in water.

8. Chloroform.

9. ONPG stock solution: O-nitrophenyl-β-D-galactoside (ONPG) is dissolved in Z-buffer (without BME) to a final concentration of 4 mg/ml and stored at −20°C.

10. 1 M Na₂CO₃.

11. Solution A with 15% glycerol (for preparation of competent cells): 10 mM MnCl₂, 50 mM CaCl₂, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.3 (pH using KOH), 15% glycerol, filter sterilize and store at 4°C.

12. Sequencing primer to sequence fusions to αCI: GCAATGAGGTGTGTCGCGTG (anneals within αCI).

13. Sequencing primer to sequence fusions to α: GTCTACGAAATGAAACCAAGC (anneals within the α gene fragment).

14. Sequencing primer to sequence reporter constructs made in pFW11 (primer 1224): CGCCAGGGTTTCCCCAGGTACCAG.

2.3.4. Equipment

1. Orbital shaker for growing bacterial cultures.

2. Roller wheel for growing bacterial cultures.


5. Heated water bath.

2.4. Protocols

2.4.1. Generation of fusion proteins

2.4.1.1. Fusions to the α subunit. Plasmid pBRxLN [7,41], which is used for making fusions to the α subunit, is derived from the medium copy number plasmid pBR322. This plasmid, which confers resistance to carbenicillin, encodes amino acid residues 1–248 of α (which corresponds to the α NTD and linker) under the control of tandem lpp and lacUV5 promoters. The lpp promoter is a strong constitutive promoter, whereas lacUV5 is IPTG-inducible. In the absence of IPTG, expression from the lacUV5 promoter is repressed by the Lac repressor. A NotI restriction site (GGGCCC) has been introduced immediately after codon 248 of α and enables DNA fragments encoding protein domains to be fused to residue 248 via a three alanine linker (GGGCCC enodes three alanine residues). A BamHI site is located immediately adjacent to the NotI site, thus enabling DNA fragments encoding protein domains to be cloned into pBRxLN on a NotI/BamHI restriction fragment.

To generate an α fusion, two PCR primers are designed to amplify the gene (or gene fragment) of interest. The 5′ end of the 5′ primer should include these additional 14 bases (TATATGCGGCCGCA) prior to the region of complementarity to the coding sequence of the protein of interest. These 14 additional bases include 5 random bases (placed at the 5′ end thus allowing the NotI restriction enzyme to efficiently digest the NotI site), a NotI restriction site, and an additional base (that allows the reading frame to be properly maintained). The 5′ end of the 3′ primer should also contain an additional 14 bases (in this case: TATATGATCTTTA) prior to the region of complementarity to the coding sequence of the protein of interest. These 14 additional bases include 5 random bases (allowing the BamHI restriction enzyme to efficiently digest the BamHI site), a BamHI restriction site, and a stop codon.

The 5′ primer and 3′ primer are used to generate a PCR product, this product is digested with NotI and BamHI and ligated into plasmid pBRxLN that has been digested with NotI and BamHI. The ligation mixture is then transformed into XL1-Blue cells and the cells are plated on LB-agar containing 100 μg/ml carbenicillin. Individual transformants are used to inoculate 5 mL overnight cultures in LB containing 100 μg/ml carbenicillin. Plasmid DNA is then isolated from the overnight cultures and individual clones are digested with NotI and BamHI to confirm the presence of a correct insert. Clones carrying an insert of the correct size are used for subsequent cloning.

2.4.1.2. Fusions to αCI. Plasmid pACCI32 [7,41], which is used for making fusions to αCI, is derived from the medium copy number plasmid pACYC184. This plasmid, which confers resistance to chloramphenicol, encodes full-length αCI under the control of the IPTG-inducible lacUV5 promoter. In the absence of IPTG, expression from the lacUV5 promoter is repressed by the Lac repressor. A NotI restriction site (GGGCCC) has been introduced immediately after codon 237 of αCI and enables DNA fragments encoding protein domains to be fused to full-length αCI via a three alanine linker. A BstYI site is located immediately adjacent to the NotI site, thus enabling DNA fragments encoding protein domains to be cloned into pACCI32 on a NotI/BstYI restriction fragment.

To generate αCI fusion, two PCR primers are designed to amplify the gene (or gene fragment) of interest. As with the primers designed to amplify DNA fragments that are to be fused to α, the 5′ end of the 5′ primer should include these additional 14 bases (TATATGCGGCCGCA) and the 5′ end of the 3′ primer should also contain an additional 14 bases (in this case: TATATGATCTTTA) prior to the region of complementarity to the coding sequence of the protein of interest. The 5′ primer and 3′ primer are used to generate a PCR product, this product is digested with NotI and BstYI and ligated into plasmid pACCI32 that has been digested with NotI and BstYI. The ligation mixture is then transformed into XL1-Blue cells and the cells are plated on LB-agar containing 25 μg/ml chloramphenicol. Individual transformants are used to inoculate 5 mL overnight cultures in LB containing 25 μg/ml chloramphenicol. Plasmid DNA is then isolated from the overnight cultures and individual clones are digested with NotI and BstYI to confirm the presence of an insert. Clones carrying an insert of the correct size are used for subsequent cloning.

2.4.2. Generating a plasmid to over-produce DNA-binding regulatory factors for use in the “one-hybrid” variation of the two-hybrid assay

When examining protein–protein interactions between a DNA-bound protein and sub-domains of RNAP fused to the αNTD the regulatory factor being investigated needs to be produced from a plasmid that is compatible with pBRxLN. I typically use pACYC184-derived vector, pAC(tac) [33], for this purpose. Plasmid pAC(tac) can be used to direct the synthesis of a protein under the control of the IPTG-inducible tac promoter. Protein-coding sequences are introduced into pAC(tac) on an Ndel/BstYI (or BamHI)
fragment. The Ndel site (CATATG) includes the start codon for the protein. 

When designing a primer to introduce a particular gene into pAC(tac) the 5’ end of the 5’ primer should include these additional 11 bases (TATATCATATG) prior to the region of complementarity to the coding sequence of the protein of interest. These 11 additional bases include 5 random bases (placed at the 5’ end thus allowing the Ndel restriction enzyme to efficiently digest the Ndel site) and an Ndel restriction site. The 5’ end of the 3’ primer should also contain an additional 14 bases (in this case: TATATGATCTTTA) prior to the region of complementarity to the coding sequence of an interest. These 14 additional bases include 5 random bases (allowing the BstYI (or BamHI) restriction enzyme to efficiently digest the BstYI site), a BstYI (or BamHI) restriction site, and a stop codon.

2.4.3. Generation of reporter strains (see Appendix Note #8)

This protocol is derived from [43]. Strain FW 102 [43] is a derivative of CSH 142 [genotype: F ara Δ(gpt-lac)S] carrying a streptomycin resistance gene (rpsL). Reporter strain FW 102 O2,2–62 [42] carries an F episome containing test promoter O2,2–62 (which consists of the lac core promoter with the λ operator, O2 upstream, centered at –62) fused to the lacZ gene. The F episome also carries a kanamycin resistance gene along with lacIq (which encodes LacI).

The following description is meant to enable researchers to generate a new reporter strain carrying an F episome containing a new artificial promoter fused to lacZ. The new artificial promoter will carry the binding site for a regulatory factor of interest placed upstream of the lac core promoter. The first step in constructing such a reporter strain involves introducing the new test promoter between the EcoRI and SalI sites of plasmid pFW11 placCons–35C (which confer resistance to both chloramphenicol and kanamycin) [31]. Plasmid pFW11 placCons–35C contains the test promoter placCons–35C located between EcoRI and SalI restriction sites (see Fig. 6). Test promoter placCons–35C consists of the core lac promoter along with an upstream ectopic “−35 element”. The EcoRI site is located upstream of the ectopic “−35 element” and the SalI site overlaps the transcription start site of the promoter (the SalI recognition site is GTGCAC; the A in this sequence is +1). In the context of this plasmid the test promoter is fused to translation signals and codons 8–212 of the lacZ gene. New test promoters can be constructed by generating a PCR product containing flanking EcoRI and SalI sites and cloning the EcoRI and SalI digested PCR product into EcoRI/SalI digested pFW11 placCons–35C. (Note that test promoters can also be generated by annealing two long complementary oligos.)

A long 5’ primer should be obtained that contains, in order from the 5’ end, two random bases (to allow the EcoRI restriction enzyme to work efficiently), an EcoRI site, the binding site for the regulatory factor being studied, and 21 bases of sequence complementary to the lac core promoter. The oligo should be designed taking into account the desired position of regulatory factor binding site. This long 5’ primer is then used in a PCR reaction that contains plasmid pFW11 placCons–35C as template and sequencing primer 1224 (which is also used to sequence reporter constructs made in pFW11; see above). The resulting PCR product is digested with EcoRI and SalI and ligated into EcoRI and SalI digested pFW11 placCons–35C. The ligation mixture is used to transform XL1-Blue cells and the cells are plated on LB-agar containing 25 μg/mL chloramphenicol and 50 μg/mL kanamycin. Individual transformants are used to inoculate 5 mL overnight cultures in LB containing 25 μg/mL chloramphenicol and 50 μg/mL kanamycin. Plasmid DNA is then isolated from the overnight cultures and individual clones are digested with EcoRI and SalI to determine the size of the insert. Clones carrying an insert of the correct size are sent for sequencing with primer 1224.

Once the desired pFW11 plasmid has been constructed, this plasmid is used to generate a reporter strain by transferring the test promoter to an F episome by homologous recombination. The desired recombination event transfers the test promoter-lacZ fusion along with only the kanamycin resistance marker to the F episome. To select for the desired recombination events, the F is transferred to a recipient strain which is then tested for sensitivity to chloramphenicol. For more details see [43]. Below is a protocol for generating reporter strains from pFW11 plasmids.

1. Prepare competent CSH 100 cells.
2. Streak FW 102 cells onto an LB-agar plate to isolate single colonies.
3. Transform the newly constructed pFW11 plasmid into CSH 100 cells, select for transformants on LB-agar plates containing 50 μg/mL kanamycin (note, do not select for chloramphenicol resistance as well).
4. (The conjugation can be performed in liquid culture or done on LB-agar plates. I will describe how to perform the conjugation on LB-agar plates; for a protocol to perform the conjugation in liquid culture see [43]) Using a toothpick or wooden stick scrape 5–10 colonies from the FW 102 plate and streak out onto a fresh LB-agar plate. Next, using a toothpick or wooden stick, scrape 5–10 colonies from the plate containing the pFW11 plasmid transformed into CSH 100 cells. Cross-streak these colonies over the streak made with FW 102 on the LB-agar plate. Let this plate incubate at 37°C for at least 8 h (it is fine to let the cells grow overnight).
5. (Using a toothpick or wooden stick scrape cells from the plate containing the FW 102/CSH 100 cross-streak and streak these onto LB-agar plates containing 50 μg/mL kanamycin, 100 μg/mL streptomycin, and 40 μg/mL X-gal (be sure to scrape cells growing in the area of overlap between the FW 102 and CSH 100 streaks). Plating on this media will select for FW 102 cells (which carry a resistance marker for streptomycin) that contain an F episome (which carries a resistance marker for kanamycin). However many of these colonies will not contain the desired F episome. Specifically, the majority (~70–90%) of these colonies will contain F episomes that are the result of a single recombination event instead of the desired double recombination event. The inclusion of X-gal in the agar plates is useful for helping to distinguish the desired class of conjugants; colonies that are blue likely carry correct F episomes.
6. Using a toothpick or wooden stick, pick individual blue colonies and streak them first onto a LB-agar plate containing 25 μg/mL chloramphenicol and second onto a LB-agar plate containing 25 μg/mL chloramphenicol and 50 μg/mL kanamycin.

Fig. 6. Shown is the sequence extending from the EcoRI site to the SalI site of plasmid pFW11 placCons–35C. The ectopic “−35 element”, the core promoter −35 and −10 elements, and the transcription start site (+1) are indicated.
containing 50 μg/mL kanamycin and 100 μg/mL streptomycin. Colonies carrying the desired F episome will fail to grow on the plate containing chloramphenicol.
(7) Make competent cells (see below) of an isolated chloramphenicol sensitive colony.

2.4.4. β galactosidase assays
Below is a general protocol derived from [7] to be used for assaying protein–protein interactions using the transcription activation-based two-hybrid assay (see Appendix Note #9).

2.4.4.1. Preparation of competent FW 102 O2–62 cells.
(1) Using a sterile toothpick or wooden stick streak FW 102 O2–62 cells onto an LB-agar plate containing 50 μg/mL kanamycin, grow at 37°C overnight.
(2) Pick a single colony and inoculate 3 mL of LB containing 50 μg/mL kanamycin, grow at 37°C overnight.
(3) Add the 3 mL culture to 200 mL of LB containing 50 μg/mL kanamycin, grow at 37°C until the culture reaches an OD600 of 0.5. Immediately place cells on ice.
(4) Centrifuge cells at 5000g (4°C); pour off supernatant. Keep cell pellet on ice.
(5) Resuspend cells in 5 mL of cold Solution A with 15% glycerol. Divide cells into 500 μL aliquots in microcentrifuge tubes, flash freeze in dry ice, store at −80°C.

2.4.4.2. Transformation of plasmid DNA into FW 102 O2–62 cells.
(1) Thaw competent cells on ice.
(2) Add 10 nanograms of both the α fusion and the plasmid carrying the CI fusion to a sterile microcentrifuge tube, place on ice for 5 min. Be sure to include appropriate negative controls (testing the CI fusion with plasmid pBrZLN and the α fusion with plasmid pAC63 CI is recommended).
(3) Add 50 μL competent FW 102 O2–62 cells to tubes containing plasmid DNA and incubate on ice for 10 min.
(4) Heat shock at 42°C for 2 min, then place tubes back on ice for 2 min.
(5) At this point cells can either be plated directly onto LB-agar plates containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, and 100 μg/mL carbenicillin or a recovery step can be done to allow efficient expression of the antibiotic resistance genes (inclusion of the recovery step yields higher numbers of transformants). If a recovery step is desired add 1 mL of LB to each tube and incubate at 37°C for 1 h. Pellet cells by centrifugation and pour off all but 100 μL of the supernatant. Use the 100 μL of remaining supernatant to resuspend the cell pellet and plate on LB-agar plates containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, and 100 μg/mL carbenicillin.
(6) Incubate plates overnight at 37°C.

2.4.4.3. β galactosidase assays.
(1) Pick single colonies from the transformation plates and place them to inoculate 3 mL of LB containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, 100 μg/mL carbenicillin, and IPTG (at concentrations ranging from 0 to 200 μM; for preliminary experiments performed at only one concentration of IPTG, 50 μM is recommended, although the optimal IPTG concentration for a given experiment should be determined empirically).
(2) Grow cultures at 37°C overnight (between 12 and 16 h).
(3) The next day use 50 μL of the overnight culture to inoculate 3 mL of LB containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol, 100 μg/mL carbenicillin, and IPTG (using the same concentration that was present in the overnight culture).
(4) Grow cultures at 37°C until the cells reach an OD600 between 0.3 and 0.7 (0.5 is ideal). Typically this takes between 90 and 120 min.
(5) Place the cultures on ice for 30 min.
(6) Place 1 mL of the culture in a cuvette to read and record the OD600 value.
(7) Set up the assay tube (in duplicate) by placing 200 μL of the culture in a small glass test tube that contains 800 μL of Z-buffer (with BME). Be sure to set up a tube that will serve as a blank using 200 μL of LB.
(8) Add 30 μL of 0.1% SDS and 60 μL of chloroform to each tube and vortex for 10 s.
(9) Allow tubes and the 4 mg/mL ONPG solution to equilibrate at 28°C in a water bath for 15–20 min.
(10) Start the assays by adding 200 μL of the ONPG solution to each assay tube, be sure to record the time at which each of the assays was started. I typically add ONPG to each tube at 5 s intervals. Mix the tubes by gently shaking or vortexing them at a low speed.
(11) When the tubes turn yellow, stop the reactions by adding 500 μL of 1 M Na2CO3. Record the time at which each reaction was stopped. The ideal range for the OD420 is between 0.6 and 0.9. It is important to try and stop each of the assays when they have reached the same OD420.
(12) Gently vortex the reaction tubes then allow them to sit at room temperature for 10–15 min to allow the chloroform and cell debris to settle.
(13) Place 1 mL of the reaction into a cuvette and read and record the OD420 and the OD550 value.
(14) β galactosidase activity is expressed as Miller Units which are calculated using the equation:

\[
\text{Miller units} = 1000 \times \left(\frac{\text{OD}_{420} - (1.75 \times \text{OD}_{550})}{t \times v \times \text{OD}_{600}}\right)
\]

Where \( t \) is the total time of the reaction expressed in minutes and \( v \) is the volume of culture used in the assay (which for this protocol is 0.2 mL).

3. Concluding remarks
I have outlined general strategies for detecting and studying protein–protein interactions involved in gene expression. The methodologies take advantage of a transcription activation-based bacterial two-hybrid assay. These assays permit the identification of targeted surfaces of RNAP and facilitate the isolation and characterization of amino acid substitutions within sub-domains of RNAP, some of which would be lethal in their natural context.

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Appendix A

Note 1 – In principle, the ability of two proteins to interact in the two-hybrid assay can be modulated by other cellular factors. This caveat must be considered when drawing a conclusion based on the detection of a stimulatory effect on reporter gene expression when a particular pair of alpha and CI fusion proteins is co-pro-
duced in reporter strain cells. In fact, transcription activation can occur as a result of a third protein acting as a “bridge” between two non-interacting protein domains fused to α and λCI (provided that the “bridging” protein can make non-mutually exclusive interactions with the proteins that have been fused to α and λCI) [44].

Note 2 – The ω subunit can be used to display protein domains that are fused either to its C-terminus or N-terminus [6]. Therefore, when it is desirable to fuse to the C-terminus of a protein instead of the N-terminus, the ω version of the two-hybrid assay can be used [6,11].

Note 3 – In many papers describing the use of the transcription activation-based two-hybrid assay, the test promoter used contains the λ operator O2 [8,10] (a site for which λCI has moderate affinity [16]). However, use of test promoters containing the λ operator O2 (a site for which λCI has higher affinity [16]) increases the sensitivity of the two-hybrid assays. Therefore, the use of reporter strain FW 102 O2–2–62 [42] is recommended.

Note 4 – As a general rule of thumb, it is advisable to conclude that two proteins interact in the two-hybrid assay if they result in at least a threefold increase in the amount of β galactosidase activity compared to that observed in assays done with negative controls. (Note that basal transcription from the test promoter will result in a certain amount of β galactosidase activity in assays containing the negative controls.) However, it is important to note that physiologically relevant interactions below this threshold have been observed using the two-hybrid assay [for example see [37]].

Note 5 – I have had success examining protein domains as large as 300 amino acids using the two-hybrid assay but have not explored using larger domains.

Note 6 – The one hybrid assay that enables the detection of the interaction between σ70 region 4 and the ectopic –35 element relies on the presence of amino acid substitution DS81 G within the σ70 region 4 moiety of the fusion protein [31]. This amino acid substitution appears to stabilize the folded structure of the tethered σ70 region 4 moiety and thus facilitates the detection of certain interactions that are at or below the threshold of detection [31,37].

Note 7 – It is currently unknown whether the α-σ region 4 fusion proteins can activate transcription from a test promoter where the orientation of the ectopic “−35 element” has been reversed, i.e., placed on the opposite strand of DNA in the opposite orientation.

Note 8 – An alternative strategy to make reporter strains has been developed that allows for more flexibility than the strategy described here [45] (e.g. this system can be used to generate libraries of test promoters). However, the resulting reporter strains are not compatible with the plasmids described here.

Note 9 – An alternative β galactosidase assay protocol has been developed that enables the assays to be performed in a high throughput fashion, thus saving a considerable amount of time. For details of this method, which requires the use of a temperature controlled plate reader, see [46].

References


