Engineering Bacterial Transcription Regulation To Create a Synthetic in Vitro Two-Hybrid System for Protein Interaction Assays

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Supporting Information

ABSTRACT: Transcriptional activation of σ54-RNA polymerase holoenzyme (σ54-RNAP) in bacteria is dependent on a cis-acting DNA element (bacterial enhancer), which recruits the bacterial enhancer-binding protein to contact the holoenzyme via DNA looping. Using a constructive synthetic biology approach, we recapitulated such process of transcriptional activation by recruitment in a reconstituted cell-free system, assembled entirely from a defined number of purified components. We further engineered the bacterial enhancer-binding protein PspF to create an in vitro two-hybrid system (IVT2H), capable of carrying out gene regulation in response to expressed protein interactions. Compared with genetic systems and other in vitro methods, IVT2H not only allows detection of different types of protein interactions in just a few hours without involving cells but also provides a general correlation of the relative binding strength of the protein interaction with the IVT2H signal. Due to its reconstituted nature, IVT2H provides a biochemical assay platform with a clean and defined background. We demonstrated the proof-of-concept of using IVT2H as an alternative assay for high throughput screening of small-molecule inhibitors of protein–protein interaction.

INTRODUCTION

Protein interactions (protein–protein, protein–nucleotide (DNA, RNA), and protein–small-molecule interactions) underlie most biological functions.1 However, we know far more about protein sequences than protein functions, owing largely to the rapid advances of next-generation DNA/RNA sequencing technologies. It is therefore highly desirable to develop next-generation protein technologies that allow rapid characterization of protein functions, especially protein interactions. Current approaches for protein interactions, for example, isothermal titration calorimetry2 and fluorescence polarization,3 often require costly instruments and extensive protein purification and labeling and therefore are time-consuming and limited to a few protein targets at one time. The cell-based genetic two-hybrid systems,4 on the other hand, have the advantages of carrying out a large number of protein interactions in each cell for selection or screening. In a typical genetic two-hybrid system, two target proteins are expressed inside the cell as hybrid proteins fused to an activation domain (AD) and a DNA(promoter)-binding domain (DB), respectively. The interaction between the target proteins recruits AD to the promoter region in the nucleus and activates the promoter-bound RNA polymerase. The issues with the genetic systems, however, are potential interferences from endogenous cellular proteins, which can lead to false negative or false positive results,4,5 toxicity of some expressed protein interactions, and accessibility to targets due to cellular membranes and efflux pumps.6

Cell-free systems in general have advantages over cell-based systems for protein function studies.7 Without the need to grow and genetically manipulate cells, proteins (including toxic proteins) can be made and tested in a few hours in cell-free systems. Without the barrier of a cell wall or membrane, a variety of conditions, such as addition of labeled or unnatural amino acids and small-molecule inhibitors, can be applied to cell-free systems.8 Cell-free split-protein systems (or protein fragment complementation assays4,9) have been developed for in vitro protein interaction studies and have additional advantages of simultaneously expressing the target proteins...
and detecting their interactions via simple reporter assays.\textsuperscript{10–12} In the absence of the protein–protein interaction, the split fragments of the reporter by themselves cannot reassemble into the active form. The interaction between two target proteins, each of which is fused to a reporter fragment, results in the reconstitution of the activity of the reporter.

In this work, we intend to create a synthetic in vitro two-hybrid system (IVT2H) from a reconstituted cell-free system. We chose the two-hybrid approach because the protein interaction in a two-hybrid system only has to bring the activation domain to the vicinity of the RNA polymerase, which can result in activation of the expression of an intact reporter. In comparison, the detection of the protein interaction in a split-reporter system requires the precise alignment of the active site residues of the split reporter, and the reconstitution of its native structure while it is fused to two interacting proteins. Even under a strong protein–protein interaction, the reconstituted split reporter can have a significantly lower activity than the intact (nonfragmented) reporter,\textsuperscript{13} suggesting that a majority of split fragments do not form the native structure. We reason that the two-hybrid system is potentially less affected by protein conformation than the split-reporter approach. We chose the reconstituted cell-free system because it has additional advantages of lacking most cellular proteins and activities, allowing in vitro reconstruction of the process of bacterial transcription initiation in the absence of other regulatory factors.\textsuperscript{14,15} Building on our previous work, we here report the creation of the first cell-free equivalent of the genetic two-hybrid systems.

\section*{RESULTS AND DISCUSSION}

Engineering Bacterial Transcription Regulation in the Reconstituted Cell-Free System. The design principle of IVT2H (Figure 1) was based on the process of transcriptional activation of $\sigma^{54}$-RNAP in \textit{Escherichia coli}, due to the similarity to the eukaryotic mode of gene activation.\textsuperscript{16} $\sigma^{54}$-RNAP forms an inactive transcriptional initiation complex on a $\sigma^{54}$ promoter ($\textit{pspA}$), which can be activated in \textit{E. coli} by the bacterial enhancer binding protein PspF.$\textsuperscript{16,17}$ PspF functions by binding to the upstream activation sequences (UAS) near the promoter and contacting the promoter-bound $\sigma^{54}$-RNAP via DNA looping stabilized by the binding of integration host factor (IHF).\textsuperscript{17,18} As a transcriptional activator, PspF is a modular protein, consisting of an N-terminal activation domain (AD), which forms a hexamer in order to activate transcription, and a C-terminal DNA binding domain (BD), which binds UAS (I and II) to facilitate the oligomerization of AD.$\textsuperscript{19}$ Like the transcription factors in the genetic two-hybrid systems,\textsuperscript{8} PspF was the basis for constructing the hybrid fusion proteins in IVT2H (Figure 1 and Figure S1B, Supporting Information). The components of IVT2H were derived from a reconstituted bacterial transcription and translation system.\textsuperscript{14} In addition to the purified \textit{E. coli} translation components and T7 RNA polymerase,\textsuperscript{15} the protein components of IVT2H included purified \textit{E. coli} RNA polymerase core enzyme (RNAP), recombinant \textit{E. coli} IHF, and RNase inhibitor. \textit{E. coli} $\sigma^{32}$ was added as a purified protein or expressed during the IVT2H reaction from a DNA construct (Figure S1A, Supporting Information, P$_{T7}$$\sigma^{32}$).

To demonstrate PspF-dependent, enhancer-specific transcription activation (Figure 1A), we expressed the full-length PspF (AD-BD) or its activation domain (AD) under T7 promoter in an IVT2H reaction containing a reporter DNA

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\caption{Design principles of IVT2H. (A) Detection of protein–DNA interaction. PspF or a hybrid fusion protein (AD-BD) is constitutively expressed under T7 promoter (T7) from an input DNA by T7 RNA polymerase (step 1). By binding to UAS, AD-BD is recruited near the $\sigma^{54}$ promoter ($\textit{pspA}$) on the reporter DNA (step 2), activating the promoter-bound $\sigma^{54}$-RNA polymerase holoenzyme ($\sigma^{54}$-RNAP) (step 3), leading to the GFP expression (step 4). (B) Binary protein–protein interaction that activates the reporter expression (IVT2H). The hybrid fusion proteins (AD-X and Y-Cro) are constitutively expressed from separate DNA constructs (step 1). The X and Y interaction and Cro binding to the Cro consensus operator sequence (2xcons) recruit AD-X and Y-Cro near the $\textit{pspA}$ promoter (step 2), thereby activating $\sigma^{54}$-RNAP (step 3), leading to GFP synthesis (step 4). (C) Binary protein–protein interaction that represses the reporter expression (reverse IVT2H). The X and Y interaction activates the anti-$\sigma^{32}$ expression on a repressor DNA (step 1). The synthesized anti-$\sigma^{32}$ inhibits the GFP expression under a $\sigma^{28}$ promoter (\textit{flIC}) by binding to the expressed $\sigma^{28}$ (step 2). The $\sigma^{28}$ protein is constitutively expressed from a separate DNA (step 3). In the absence of anti-$\sigma^{32}$, $\sigma^{28}$ forms an $\sigma^{28}$-RNA polymerase holoenzyme on the \textit{flIC} promoter to mediate the GFP expression (step 4). (D) Detection of protein–RNA interaction (three-hybrid IVT2H). AD-X and Y-Cro are coexpressed with a RNA substrate gene from separate DNA constructs (step 1 and 2). The interactions of the RNA substrate (bold hairpins) with both X and Y results in a three-part protein–RNA complex bound upstream to the $\sigma^{54}$ promoter ($\textit{pspA}$) on the reporter DNA (step 3). The subsequent activation of $\sigma^{54}$-RNAP (step 4) leads to the GFP synthesis (step 5).}
\end{figure}
pspA-Fluc expressing firefly luciferase (Fluc) under the pspA promoter17 with UAS I and II (Figure 1A and Figure S1B,C, Supporting Information) and measured the luciferase activities of aliquots after incubation at 37 °C. The expression of PspF resulted in a significant luciferase activity, whereas in the absence of the DNA binding domain, no significant luciferase activity was observed (Figure 2A, PspF and AD, gray columns). The data suggest that we could fuse a different DNA binding domains to the activation domain of PspF and activate transcription simply by inserting a corresponding DNA recognition sequence upstream of the promoter. Two copies of the Cro consensus sequence in 2xcons-Fluc seemed to be sufficient to induce the hexamer formation of AD to activate transcription, presumably by recruiting AD-Cro near the promoter and increasing the local concentration of AD. Use of one copy of the Cro consensus sequence decreased the reporter expression, whereas more copies of the Cro consensus sequences did not further increase the reporter expression (Figure S2A, Supporting Information). Taken together, these results not only demonstrate that we have recapitulated the PspF-mediated transcription activation in vitro but also suggest that IVT2H could potentially be used to study protein–DNA interaction (Figure 1A), a concept well-known as the yeast and bacterial one-hybrid systems.22,23

Protein–DNA Interaction. To further demonstrate the above application, we replaced Cro with the DNA binding domain of zinc-finger protein Zif268 (Zif).24 Zif binds to its consensus operator sequence (zif) as a monomer with $K_d = 0.5–5$ nM.25,26 In comparison, Cro binds to its consensus operator sequence with a much higher affinity ($K_d = 1.2$ pM).20 We used four copies of Zif consensus operator sequence as UAS to construct a reporter DNA 4xzif-GFP, expressing a green fluorescent protein for the hybrid fusion protein AD-Zif (Figure S1C, Supporting Information). To facilitate the comparison, we constructed 2xcons-GFP for AD-Cro. The expression of AD-Cro resulted in a significantly higher GFP fluorescence from 2xcons-GFP than AD-Zif from 4xzif-GFP, suggesting that the higher DNA binding affinity led to stronger transcription activation (Figure 2B, first and third columns). However, it has been postulated that the high affinity of Cro is due to its dimerization coupled to DNA binding,21,27 whereas Zif binds DNA as a monomer without the cooperativity.28 We therefore suspected that the high fluorescence signal of AD-Cro could be the synergistic effect of Cro dimerization and DNA binding on enhancing the hexamer formation of AD and transcription activation. To address this question, we fused AD to a Cro monomeric mutant Cro K56[DGEVK]. The expression of the hybrid fusion protein AD-Cro K56[DGEVK] resulted in a drastic decrease in the fluorescent signal from 2xcons-GFP (Figure 2B, second column) compared with AD-Cro and to a similar level as that of AD-Zif from 4xzif-GFP (Figure 2B, third column). The data are consistent with previous studies suggesting that the K56[DGEVK] mutation reduced Cro DNA binding affinity by >2000-fold.28 Taking a step further, we added a synthetic leucine zipper (AA4)29 to the C-terminus of Zif to artificially homodimerize Zif. The expression of the resulting hybrid fusion protein AD-ZifAA4 indeed increased GFP fluorescence significantly compared with AD-Zif from the same reporter DNA (4xzif-GFP) (Figure 2B, fourth and third columns). As control experiments, AD-Cro K56[DGEVK] or AD-Zif was expressed in IVT2H with a reporter DNA containing a nonspecific UAS (4xAT-GFP, Figure S1C, Supporting Information). Both hybrid fusion proteins generated significantly higher GFP fluorescence from the specific UAS (2xcons and 4xzif, respectively) than from the nonspecific UAS (Figure S2B, Supporting Information, white vs gray columns).
**Protein–Protein Interaction.** To demonstrate real-time detection of protein–protein interaction in IVT2H (Figure 1B), we first chose FK506 binding protein (FKBP) and FKBP-rapamycin binding domain of mTOR (FRB), known to form a heterodimer in the presence of rapamycin. The hybrid fusion proteins, AD-FKBP and FRB-Cro were coexpressed in the IVT2H reactions containing the reporter DNA 2xcons-GFP in the presence or absence of rapamycin. The fluorescent signal of each reaction was monitored in real-time during incubation at 37 °C. In the presence of rapamycin, GFP fluorescence increased significantly after ∼60 min and reached a saturation after ∼6 h (Figure 3A, left panel, +rapamycin). In the absence of rapamycin, only a small increase in the GFP fluorescence was observed over a period of 8 h (Figure 3A, left panel, −rapamycin). Here we successfully demonstrated the concept of the genetic two-hybrid system in IVT2H, whereby the specific interaction between FKBP and FRB from expressed hybrid fusion proteins resulted in transcription activation and GFP expression. Using FKBP and FRB, we further demonstrated the use of IVT2H as a reverse two-hybrid system in which the protein–protein interaction represses the reporter expression (Figure 1C). In the reverse IVT2H, the FKBP and FRB interaction was designed to activate the expression of an E. coli transcription repressor anti-σ, which inhibits the GFP expression under a σ promoter flic by binding to a coexpressed σ (Figure 1C). In addition to the DNA constructs for AD-FKBP and FRB-Cro, the reverse IVT2H reaction contained a repressor DNA (2xcons-anti-σ), a σ, expressing DNA (PT7-σ), and a reporter DNA (flic-CFP) (Figure S1A, Supporting Information). In contrast to the IVT2H reaction (Figure 3A, left panel), the presence of rapamycin in the reverse IVT2H reaction resulted in a low GFP signal (Figure 3A, right panel, +rapamycin); whereas the absence of rapamycin led to a significant increase in the GFP signal (Figure 3A, right panel, −rapamycin).

To further establish IVT2H for detecting binary protein–protein interactions, we tested a number of other protein pairs with a wide range of known affinities (Table S1, Supporting Information). We observed a remarkable correlation of the intensity of GFP fluorescence with the reported Kd of the protein–protein interaction (Figure 3B, between 10 and 1000 nM). However, no obvious correlation was observed when Kd is below 10 nM (Figure 3B, between 0.1 and 10 nM). The data suggest that the affinity of the binary protein–protein interaction, if between 10 nM and 1.0 μM, primarily determined the amount of the synthesized reporter protein under the IVT2H conditions. Despite the dynamic nature of the IVT2H reaction in which the concentrations of the synthesized proteins change over time and are limited by the overall protein synthesis capacity, the correlation of the protein interaction affinity with the signal output in IVT2H can potentially be described by a mathematical model for three-component binding equilibria. The formation of a ternary complex of AD-X, Y-Cro, and the reporter DNA in IVT2H is a critical step for transcription activation (Figure 1B) and therefore is directly correlated to the GFP expression. In the equilibria of three components, AD-X, Y-Cro, and the reporter DNA, the concentration of the ternary complex is determined by the concentration of each component, the affinity between X and Y (Kd) and the affinity between Cro and its specific UAS (KcDNA) (Figure S2C, Supporting Information). Cro binds its UAS with a Kd of 1.2 pM, making the affinity of X and Y a limiting factor for the ternary complex formation. Using simulations provided by the mathematical model, we plotted the dose–response curves of the ternary complex at different affinities of X and Y (Figure S2D, Supporting Information). The results (see the legend in Figure S2D, Supporting Information) are consistent with not only the observed correlation between the GFP expression and the affinity of the protein–protein interaction (between 10 and 1000 nM, Figure 3B) but also with the observed noncorrelation at affinities below 10 nM (between 0.1 and 10 nM, Figure 3B).

**Protein–RNA Interaction.** We further demonstrated the use of IVT2H as a three-hybrid system for the detection of protein–RNA interactions (Figure 1D). As the hybrid fusion proteins, we chose the first 22 residues of the N-protein of the bacteriophage lambda (λN22) and the coat protein of the Pseudomonas phage PP7 (PP7CP), generating AD-λN22 and PP7CP-Cro, respectively. Since λN22 and PP7CP bind to their hairpin RNA substrates, boxB and PP7, respectively, we created a substrate RNA construct (PboxB-PP7), which can produce a hybrid RNA substrate (λboxB-PP7) from T7 promoter during the IVT2H reaction. As a control, PP7 was replaced by TAR, a hairpin RNA from HIV, to generate a nonsubstrate construct (PboxB-TAR). In the IVT2H reactions expressing both AD-λN22 and PP7CP-Cro, addition...
of P\textsubscript{T\textasciitilde} \lambda boxB-PP7 resulted in significant luciferase activity (Figure 4, third column), whereas in the absence of P\textsubscript{T\textasciitilde} \lambda boxB-

Figure 4. Detection of protein–RNA interaction in three-hybrid IVT2H. The hybrid fusion proteins AD-\lambda N22 and PP7CP-Cro were expressed in the IVT2H reactions in the presence or absence of the substrate RNA construct (P\textsubscript{T\textasciitilde} \lambda boxB-PP7) or the nonsubstrate control (P\textsubscript{T\textasciitilde} \lambda boxB-TAR). The data are means from at least two independent IVT2H reactions; error bars show sd.

PP7 or the presence of the nonsubstrate RNA (P\textsubscript{T\textasciitilde} \lambda boxB-TAR), no significant luciferase activity was observed (Figure 4, first and second columns, respectively). Expression of only one hybrid protein (AD-\lambda N22) also failed to generate significant luciferase activity (Figure 4, fourth column). The data suggest that the specific binding of the hybrid fusion proteins to both RNA substrates activated the expression of the luciferase reporter.

**Screening Small-Molecule Inhibitors of Protein–Protein Interaction.** In vitro biochemical assays and genetic two-hybrid systems have been widely used for high-throughput screening (HTS) of small molecule inhibitors of protein–protein interactions.\textsuperscript{36,37} To demonstrate the use of IVT2H as a homogeneous mix-and-read biochemical assay for HTS, we chose the protein–protein interaction between the ligand binding domain of human estrogen receptor \(\alpha\) (ER\(\alpha\) LBD) and the receptor interaction domain of human nuclear receptor coactivator 1 (NCOA1 RID), due to commercial availability of small-molecule modulators and the fact that the steroid hormone 17\(\beta\)-estradiol (E2) can induce the interaction by binding to ER\(\alpha\) LBD.\textsuperscript{38} We first showed that addition of E2 resulted in a dose-dependent increase in the luciferase activity (Figure S3A, Supporting Information), thus establishing IVT2H as an in vitro assay for the ER\(\alpha\)/NCOA1 interaction. Using such IVT2H assay with E2, we set up to screen a library of 67 steroid and steroidal mimic compounds (Table S2, Supporting Information) for inhibitors of the ER\(\alpha\)/NCOA1 interaction in a 1536-well plate quantitative high-throughput screen (qHTS) format.\textsuperscript{39} The activity of each compound was determined over an 11-point titration range from nanomolar to micromolar range at the end of the IVT2H reactions (Figure S3, Supporting Information). This could explain that higher IC\(_{50}\) values were obtained in the IVT2H assays than those of biochemical inhibition assays in which the protein concentrations are often fixed at a low nanomolar range.\textsuperscript{42} Nevertheless, the rank order of inhibition observed in the IVT2H assay for these compounds seemed to be consistent with other assays (Figure S5B).\textsuperscript{40,41}

In summary, we recreated the process of bacterial enhancer-specific gene activation in a reconstituted cell-free system using a bottom-up synthetic biology approach. We further engineered such synthetic system to create IVT2H and demonstrated its broad utility as a universal assay format for protein interactions. Unlike cell-based genetic systems that require manipulating cellular genetic backgrounds for detecting different types of protein interactions, IVT2H can be formulated into one-hybrid, two-hybrid, reverse two-hybrid, or three-hybrid assay simply by changing DNA constructs, while the protein components of IVT2H remain unchanged. Such flexibility of IVT2H can be
harnessed for more applications. For instance, IVT2H can be constructed as a reverse two-hybrid assay for screening small-molecule inhibitors, allowing inhibition to generate a positive signal (Figure S3B, Supporting Information). As a three-hybrid system, IVT2H can potentially be used to detect not just RNA but any molecule in solution (Figure 1D).

Detection of protein interactions in IVT2H requires the same reconstituted protein translation machinery to synthesize multiple proteins (interacting proteins, σ54, and reporter protein) in coupled steps. In in vitro reactions, IVT2H has a defined capacity for protein synthesis; therefore, the amounts of DNA in IVT2H affect the optimal signal-to-noise ratio. Since the interacting proteins and σ54 are expressed under the strong T7 promoter and the reporter under the relatively weak σ54 promoter, the DNA concentrations in IVT2H are adjusted to the picomolar range for the interacting proteins and σ54 and the nanomolar range for the reporter. By varying the DNA concentrations, we determined that the optimal DNA concentration for the DNA-interacting protein is ~10 pM and that for protein–protein interacting pairs is ~50 pM (Figure S4A,B, Supporting Information). Higher DNA concentrations resulted in higher amounts of the interacting proteins (Figure S5A,B, Supporting Information) but lower GFP reporter signals (Figure S4A,B, Supporting Information), likely due to the limited synthesis capacity of IVT2H. The optimal DNA concentrations and the correlations of the DNA concentrations with the protein amounts in IVT2H have been carefully characterized for only a few proteins (Figure S5, Supporting Information). It is possible that some interacting proteins are not expressed or are expressed at unusually high levels, which could lead to false negative or false positive signals in IVT2H. At least 4.4–10 nM of the reporter DNA is needed in IVT2H to generate sufficient GFP signal (Figure S4C, Supporting Information). Further increasing the reporter DNA concentrations can result in a stronger GFP signal but also a higher signal from the nonspecific interaction (Figure S4C, Supporting Information). In addition, using higher reporter DNA concentrations increases the cost of making DNA. The protein components of IVT2H can be readily purified or obtained from commercial sources (Table S4, Supporting Information).

Cell-free split-protein systems represent a similar approach to IVT2H for in vitro detection of a variety of protein interactions.10,11 Like IVT2H, split-protein systems can also be applied for screening small-molecule inhibitors of protein–protein interaction.44 The signal from the cell-free split-protein assay is dependent on the reconstitution of the activity of the reporter protein, potentially can be monitored in real-time, but is not coupled to a gene activation event (at least in vitro). In IVT2H, protein interaction is coupled to the activation of a reporter gene via domain recruitment. Consequently the signal is amplified by the transcription and translation of the nonfragmented reporter protein. In addition, we show that the signal from IVT2H is correlated to the relative strength of the protein interaction.

CONCLUSIONS

Though a variety of genetic systems and in vitro systems for detecting protein interactions have been developed,4 direct comparison of these methods against the same reference sets reveals that each method has inherent limitations and often detects a subset of interactions.45 In this work, we develop IVT2H as a unique cell-free system for detection of protein interactions, which is clearly distinct from cell-based methods and cell-free split-protein systems. IVT2H may serve as an alternative and independent tool for validating existing protein interaction data and providing new ones. Without using cells, IVT2H may detect “elusive” protein interactions, such as those of difficult-to-express proteins or those toxic to cells. The next step for IVT2H is its integration with high-throughput platforms to allow selection or screening of protein interactions, a current advantage of cell-based genetic systems. Cell-free systems are compatible with liposomes and microdroplets; thus use of IVT2H for high throughput protein interaction studies is highly possible.

As a reconstituted synthetic biological system, IVT2H contains a defined number of components with known initial concentrations, thus representing a unique experimental model for computational simulations of synthetic gene circuits or chemical biology of gene regulation.

MATERIALS AND METHODS

Reconstitution of IVT2H. IVT2H was based on previous work that coupled E. coli transcriptional machinery to the reconstituted protein synthesis system.18,19 IVT2H typically contained the reconstituted protein synthesis system (T7 RNA polymerase),14,15 purified E. coli RNA polymerase core enzyme (New England Biolabs), purified recombinant E. coli IFH,43 murine RNase inhibitor (New England Biolabs), and DNA constructs (plasmids and linear DNA) expressing sigma factors, hybrid fusion proteins, RNA substrates, and reporters (see below).

DNA Constructions for IVT2H. The gene for E. coli sigma S4 (σ54) was amplified by PCR from E. coli genomic DNA and cloned into an expression vector pCOAT containing a T7 promoter48 to generate the plasmid DNA (Figure S1A, Supporting Information). The σ54 protein was expressed in E. coli from P15C-σ54 as a recombinant protein with a N-terminal 6xhis tag and purified according a previous protocol.48,49 The gene for E. coli phage-shock protein F (PspF) was amplified from E. coli genomic DNA and cloned into pCOAT to give P15C-PspF (Figure S1B, Supporting Information). Similarly, the activation domain (AD, residues 1–296) of PspF was cloned into pCOAT to give P15C-AD. The gene for the lambda repressor protein Cro49 was amplified from lambda phage DNA, whereas the genes for Cro K56[DGEVK],50 the zinc finger DNA binding domain of Zif268 (Zif),51 and the fusion protein ZifAA52 were synthesized commercially (Integrated DNA Technologies) (Figure S1B, Supporting Information). The genes for interacting protein pairs (X and Y), including FK506 binding protein (FKBP) and a 100-amino acid domain (E2015 to Q2114) of the mammalian target of rapamycin (mTOR) known as the FKBP-rapamycin binding domain (FRB),52 the first 22 residues of the N-protein of the bacteriophage lambda (IN22),53 and the coat protein of the Pseudomonas phage PP7 (PP7CP),54 and the protein pairs listed in Supplementary Table 1, Supporting Information, were synthesized commercially (Integrated DNA Technologies) and typically cloned to the C-terminus of AD in pCOAT to generate P15C-AD-X, or the N-terminus of Cro in pCOAT to generate P15C-Y-Cro (Figure S1B, Supporting Information). To generate the RNA substrate for the three-hybrid IVT2H, the gene for the hybrid hairpin RNA zboxB-PP7 was synthesized and cloned into pUCA105T7 to generate the substrate DNA construct (P15C-zboxB-PP7). As a control, PP7 was replaced by TAR, a hairpin RNA from HIV,55 to generate a nonsubstrate DNA (P15C-zboxB-TAR).

To construct a reporter DNA for σ54-mediated transcription activation, the −130 to +20 region of the E. coli pspA promoter was amplified from E. coli genomic DNA to replace the T7 promoter in pUCA108T7.14 The gene for firefly luciferase (fluc) was then cloned into such vector to generate pspA-Fluc (Figure S1C, Supporting Information). The pspA promoter region contains a binding site for IFH and two upstream activation sequences (UAS I and II, between position −80 and −126 relative to the transcription start site) for
PgpF. To facilitate the replacement of UAS I and II with other DNA recognition sequences, we introduced a unique restriction enzyme site (BamHI) at 3′ end of UAS II such that UAS I and II were flanked by BstNI and BamHI sites. By simple linker insertion, we replaced UAS I and II with the consensus operator sequence for Cro (consensus) or Zif (zif) (Figure S1E, Supporting Information). As a non-specific DNA binding control (nonspecific UAS), we also replaced UAS I and II with an arbitrary AT-rich sequence (AT) (Figure S1E, Supporting Information). To allow real-time monitoring of transcription activation, we also cloned the gene for green fluorescence protein (GFP) in the reporter DNA constructs, replacing Fluc as the reporter (Figure S1C, Supporting Information). In all IVT2H reactions, the reporter DNA constructs were used as linear PCR fragments generated by direct amplification from the corresponding plasmids using the primer pUCAw (5′-CAGGGTTATTGTCTCAT-GAGCGG-3′) and pUCArv (5′-GAGCTGATACCGCTCGCCG-CAGC-3′).

For the reverse IVT2H, the gene for E. coli sigma 28 (σ28) was amplified by PCR from E. coli genomic DNA and cloned into pCOAT to generate Pσ28-σ28 (Figure S1A, Supporting Information). The gene for E. coli anti-sigma 28 (anti-σ28 or FglM) was amplified by PCR from E. coli genomic DNA to replace the Fluc gene in 2xcons-GFP, generating the repressor DNA 2xcons-anti-σ28 (Figure S1D, Supporting Information). To construct the reporter DNA for σ28-mediated transcription activation, −120 to +20 region of the E. coli fliC promoter5 was amplified from E. coli genomic DNA to replace the T7 promoter in pUCA108T7.4 For the gene for Fluc or GFP was then cloned into this vector to generate fliC-Fluc or flic-GFP (Figure S1C, Supporting Information).

Setting up IVT2H Reactions. Unless specified otherwise, all IVT2H reactions contained 144 nM RNA polymerase core enzyme, 1.2 μM IHF, 0.8 units μL−1 RNAase inhibitor, 0.2 ng μL−1 (45 pM) plasmid DNA expressing σ28, 0.2 ng μL−1 (~40–60 pM) DNA constructs expressing hybrid fusion proteins, 4.4 nM linear reporter DNA expressing Fluc or GFP, and the reconstituted protein synthesis system (Table S4, Supporting Information). For binary protein–protein interactions in Figure 3B, purified recombinant σ28 protein (80 nM) was used instead of the plasmid DNA (although no major difference was observed between protein and DNA). For the reverse IVT2H (Figure 3A, right panel, and Figure S3B, Supporting Information), the plasmid DNA Pσ28-σ28 (40 pM), the plasmid reporter DNA flic-GFP or fluc-Fluc (1.5 nM) and the linear repressor DNA 2xcons-anti-σ28 (4.4 nM) were additionally used. For the three-hybrid IVT2H (Figure 4), the plasmid DNA Pσ28-AboxB-P7 (80 pM) was additionally used. For the IVT2H reactions involving small molecules (except those for qHTS), small molecule stocks (in H2O or DMSO (<25%)) were added (typically 1 μL in a 25 μL reaction) in the indicated final concentrations before the IVT2H reactions. For the IVT2H involving the interaction between phosphorylated YEEI peptide (pYEEI) and SH1 domain (Figure 3B, Table S1, Supporting Information), purified recombinant Src kinase (GST-tagged human Src kinase from Sigma; 1.5 μL in a 25 μL reaction) was added before the reaction.

IVT2H reactions were typically set up by mixing the DNA constructs with the protein components in a 25 μL reaction volume and then incubating at 37 °C in 500 μL Eppendorf tubes for the luciferase reporter or in a 383-well microplate (Corning, Lowell, MA) for the GFP reporter. IVT2H for qHTS is described separately below.

Activity Assays of the Reporter Proteins. The activity of the luciferase reporter (Fluc) was assayed using the Luciferase Assay System (Promega, Madison, WI) in a microplate luminometer (Centro LB 640, Berthold Technologies, Oak Ridge, TN) according to manufacturers’ instructions. Aliquots (5 μL) from the IVT2H reactions incubated at 37 °C for 2–4 h were diluted 10-fold in 1x cell culture lysis reagent (Promega, Madison, WI) containing 1 mg/mL BSA. Aliquots (5 μL) of the dilutions were then added in triplicate to a microplate for the luciferase assay in the luminometer. The GFP fluorescence (ex. 513 nm, em. 532 nm) was measured directly from a 384-well microplate (Corning, Lowell, MA) every 5 min for up to 8 h in a Spectramax M5 microplate reader ( Molecular Devices, Sunnyvale, CA) in which the chamber temperature was maintained at 37 °C.

Using IVT2H for qHTS Assay. qHTS is the simultaneous testing of compound arrays across a dilution series covering 4–5 orders of magnitude in concentration. The approach, most efficient in low-volume assay formats, enables the pharmacological characterization of chemical libraries. To adapt the IVT2H assay to a qHTS format, a 1536-well white/solid bottom plate (Cat. no. 789175-F, Greiner Bio-One North America) was pretreated with 5 μL of 0.1% tween-20 using the BioRAPTR flying reagent dispenser (FRD; Beckman Coulter Inc., Indianapolis, IN), covered, and allowed to stand overnight at ambient temperature. Next day, the plate was inverted and centrifuged for 3 min at 1000 rpm to remove detergent and allowed to air-dry. Two microliters/well of IVT2H solution (final concentrations 60% ER-NR-Coa1 solution A, 40% PIA solution B) with or without 20 nM β-estradiol (E2) were dispensed into respective wells of the pretreated plate with a BioRAPTR FRD where the dispensing tube had been precoated with 0.1% tween-20 to prevent adsorption of protein components. A 1536-well compound library plate (Cat. no. 789270-C, Greiner Bio-One North America) containing 11-point titrations of 67 steroids and steroid-mimetics from the Prestwick collection (Prestwick Chemical, Washington, DC) was prepared in DMSO starting at concentration between 4.5 and 10 mM and ending with a concentration of between 75.7 and 169 nM, depending on the specific compound (see Figure 3). From this plate, 23 nL of each sample was transferred using a 1536-well pin tool (Wako HorNet-KL uHTS system, DJK Scientific, Inc., San Diego, CA), which allows for delivery of each compound to the assay plate at final compound concentrations ranging from approximately 51–115 μM at 87 pm to 2 nM. Controls were as follows: 23 nL/well of high concentration (final concentration 115 μM) 4-hydroxytamoxifen positive antagonist control, a 16-point 1:2 titration (115 μM to 3.5 nM final concentration range) of 4-hydroxytamoxifen in duplicate/plate control titration, or DMSO vehicle control were dispensed into columns 1, 2, 3, and 4 respectively, with the Wako pin tool as above. The assay plate was manually covered with an aluminum foil plate seal (Cat. no. 6569, VWR, Bridgeport CT), centrifuged for 30 s at 1000 rpm, and incubated at 37 °C for 4 h. One microliter/well of One-Glo Fluc reagent (Promega Corporation, Madison, WI) was added to each well of the assay plate with a BioRAPTR FRD. The plate was incubated for 10 min at room temperature, protected from light, and luminescence was measured with a ViewLux uHTS Microplate Imager (PerkinElmer, Waltham, MA) with a clear emission filter, 1 s exposure, medium gain, slow speed, and 2 × 2 binning.

Concentration–Response Curve Fitting. qHTS data was displayed after correction for plate-based aberrations and normalization using Origin software (OriginLab Corp., Northampton, MA) for the three-axis plot of activity for the 67 compounds (Figure 5A) or in Prism (GraphPad Software, San Diego, CA) for the four inhibitors (Figure S8).

ASSOCIATED CONTENT

1 Supporting Information
Supporting data and figures, details of DNA constructs, protocols, and lists of protein interaction pairs, HTS compounds, and components of IVT2H. This material is available free of charge via the Internet at http://pubs.acs.org.

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