Receptor-Dependence of the Transcription Read-Out in a Small-Molecule Three-Hybrid System

Wassim M. Abida,[a, b] Brian T. Carter,[a] Eric A. Althoff,[a] Hening Lin,[a] and Virginia W. Cornish*[a]

Small-molecule three-hybrid systems show promise as an in vivo alternative to affinity chromatography for detecting small-molecule – protein interactions. While several three-hybrid systems have been reported, little has been done to characterize these systems and, in particular, to test the assumption that the protein – small-molecule interaction can be varied without disrupting the transcription read-out. Recently we reported a dexamethasone – methotrexate chemical inducer of dimerization (CID) for use in the yeast three-hybrid system, based on the well-studied ligand – receptor pairs dexamethasone (Dex) – glucocorticoid receptor (GR) and methotrexate (Mtx) – dihydrofolate reductase (DHFR). Here we describe our first efforts to characterize this system, by focusing on a comparison of the activity of a bacterial and a mammalian DHFR as a test case of the influence of the ligand – receptor pair on the transcription read-out. By using a lacZ *reporter gene, the activity of several GR and DHFR protein chimeras with different orientations and linker sequences and Dex – Mtx CIDs with different chemical linkers have been compared. In addition, Western analyses and in vivo biochemical assays have been carried out to confirm the integrity of the GR and DHFR protein chimeras. The transcription read-out is found to be much more sensitive to the structure of the protein chimeras than the CID. The most surprising result is that the levels of transcription activation are consistently higher with the bacterial than the mammalian DHFR, despite the fact that both proteins bind Mtx with an inhibition constant (K_i) in the low pM range. These results set the stage for understanding three-hybrid systems at the biochemical level so that they can be used to detect ligand – receptor pairs with a range of structures and dissociation constants.*

KEYWORDS:

molecular recognition · protein engineering · reductases · small-molecule – protein interactions · three-hybrid assay

Introduction

Recently we reported a dexamethasone (Dex) – methotrexate (Mtx) chemical inducer of dimerization (CID) for use in the yeast three-hybrid system.[1] In the small-molecule three-hybrid system a dimeric ligand bridges a receptor – DNA-binding-domain (DBD) protein chimera and a receptor – activation-domain (AD) protein chimera, effectively reconstituting a transcriptional activator and increasing transcription of a downstream reporter gene (Figure 1). Three-hybrid systems show promise for the detection of small-molecule – protein interactions by varying one of the ligand – receptor pairs; they may be of use to identify the in vivo targets of drugs, to evolve new ligand – receptor pairs, or to screen complementary DNA (cDNA) libraries based on function.[2–4] The advantage of the three-hybrid system should be its compatibility with entire cDNA libraries or large libraries of protein variants. While several three-hybrid systems have been reported, little has been done to characterize these systems and understand the influence of the CID and protein-chimera structure on the transcription read-out.[5–9] At the outset, we wanted to characterize our Dex – Mtx yeast three-hybrid system and, in particular, test the assumption that the ligand – receptor pairs (one half of the CID) can be varied without affecting transcription activation.

One advantage to using Mtx as a CID is that the interaction between Mtx and dihydrofolate reductase (DHFR) is very well

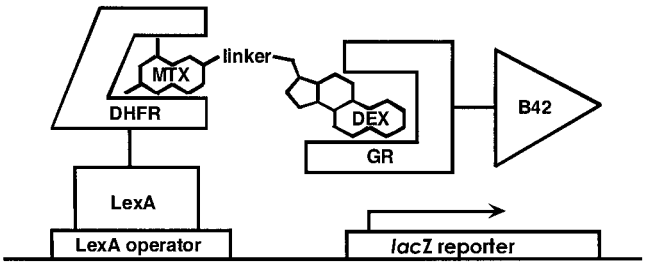

Figure 1. *Yeast three-hybrid assay. A binding site with high affinity for the DNA-binding protein LexA is placed upstream of a* lacZ *reporter gene, which encodes β-galactosidase. A dexamethasone – methotrexate (Dex – Mtx) heterodimer bridges a LexA – dihydrofolate reductase (LexA – DHFR) and a B42 – glucocorticoid receptor (B42 – GR) fusion protein. Thus, the Dex – Mtx small molecule effectively reconstitutes the LexA – B42 transcriptional activator, increasing transcription of the* lacZ *reporter gene and production of β-galactosidase.*

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characterized and so lends itself to biochemical studies.[10–13] It is well established that while both bacterial and mammalian DHFRs are inhibited by Mtx with affinity in the low pM range, their kinetics and dependencies of binding on nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) differ. Thus, we reasoned that a comparison of the behavior of a bacterial and a mammalian DHFR in our system would provide a simple case study of the influence of the ligand – receptor pair on the transcription read-out. In our original system we used the DHFR from *Escherichia coli* (eDHFR). For the mammalian DHFR, we chose the murine DHFR (mDHFR) because it is one of the better characterized DHFRs and because it has been used extensively for studies of ubiquitin-mediated protein degradation in *Saccahromyces cerevisiae*.[14] While their primary sequences have diverged and the two proteins have only 29% identity, the overall three-dimensional structures of the two proteins are quite similar. The two proteins are thought to have similar, conserved active-site residues based both on biochemical studies and high-resolution structures of several complexes of eDHFR and a close homologue of mDHFR.[11, 13, 15, 16] Both proteins are monomeric, and the inhibition constant (K_i) values of Mtx for both proteins are indistinguishable, 1 – 20 pM depending on experimental conditions.[17–21] Binding of Mtx to both proteins follows a two-step mechanism, with isomerization to the second, high-affinity complex being the rate-determining step. The individual rate constants for both steps, as well as their NADPH dependencies, have been determined for the bacterial as well as mammalian DHFRs. The advantage of comparing different species of the same receptor, as opposed to different ligand – receptor pairs, is that one controls for differences in ligand solubility and the orientation of the ternary complex.

Based on previous work with yeast two- and three-hybrid systems, we wanted to compare the activity of these two receptors in different contexts. There are surprisingly few studies on the influence of the CID linker on the efficiency of protein dimerization and transcription activation. It is likely that this is because initial reports suggested that the CID linker had little influence on the biological read-out and considerable effort is required to synthesize many of these molecules.[5] There are reports, most notably with the α-keto-pipecolyl-amide FK506 analogues, where the linker strongly affects the levels of transcription activation.[9, 22, 23] Thus, we wanted to compare the activity of the DHFR proteins with Dex – Mtx CIDs with different chemical linkers. Work with the two-hybrid system for detecting protein – protein interactions has shown that the nature of the protein chimeras can dramatically influence the activity of the reconstituted transcriptional activator. This has been reported both in individual two-hybrid screens and in a systematic study of the relation between the dissociation constant (K_D) of a protein – protein interaction and the level of transcription activation by Estojak et al.[24] For example, the interaction between Max and Myc, two eukaryotic helix – loop – helix proteins known to form heterodimers, could only be detected when Max was fused to the DBD and Myc to the AD, but not when the orientation was reversed. Thus, we tested several different DHFR and glucocorticoid receptor (GR) chimeras.

In our initial Dex – Mtx three-hybrid system, a Dex – Mtx CID with a *m*-xylene linker bridged an *E. coli* DHFR – DBD and a GR – AD fusion protein, thereby activating transcription of a *lacZ* reporter gene. Here we have characterized this system by comparing the levels of transcription induced by Dex – Mtx with *E. coli* DHFR and murine DHFR. To ensure that any differences were not simply due to the small-molecule heterodimerizer, we synthesized Dex – Mtx CIDs with different chemical linkers and tested the levels of transcription activation with these different CIDs. Because previous studies with the two-hybrid system have shown that the read-out can be protein-chimera dependent, we also varied the structures of the DHFR and GR protein chimeras. Finally, to begin to understand the basis for the difference in activity between the *E. coli* and murine DHFRs, we determined the expression levels and in vivo activity of the DHFR fusion proteins.

Results

Transcription activation by the bacterial and mammalian DHFRs differs significantly

We began by comparing the levels of transcription induced by Dex – Mtx with the *E. coli* and murine DHFRs in our original system with DBD – DHFR and AD – GR fusion proteins and a *lacZ* reporter gene (see Figure 1).[1] This system is based on the well-characterized ligand – receptor pairs dexamethasone – glucocorticoid receptor[25] and methotrexate – dihydrofolate reductase.[11] It employs the Brent two-hybrid system where the DNA-binding domain is the bacterial protein LexA, the activation domain is the artificial B42 activator isolated from a library of *E. coli* genomic DNA, and the reporter is the *lacZ* gene under control of eight tandem LexA operators.[26] Specifically, either the *E. coli* or murine DHFR, a variant of the hormone-binding domain of the rat GR with two point mutations, and a Dex – Mtx CID with a *m*-xylene linker (D5M) were employed.

Using standard β-galactosidase activity assays both on plates and in liquid culture,[27] we compared the levels of transcription activation induced by D5M in the presence of LexA – eDHFR or LexA – mDHFR (Figure 2, lanes 3 and 4; Figure 3B). β-Galactosidase levels were estimated based on enzyme-catalyzed hydrolysis of the chromagenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in the plate assays or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) in the liquid assays. For the liquid assays, the cells were lysed, and the levels of ONPG hydrolysis were normalized based on total protein concentrations determined by a Bradford test. In the β-galactosidase assays, the Dex – Mtx CID is simply added along with the other components to the cell media, and the extracellular concentrations of the CID ranged from 10 nM to 10 μM. For both the plate and the liquid assays, the cells were incubated in the presence of the CID for 2 – 5 days. Control assays established that transcription activation was dependent on Dex – Mtx (Figure 2). As reported originally, Dex – Mtx-activated transcription could only be competed out with Mtx, not with Dex (Figure 2 and data not shown). Transcription activation, however, was always abrogated when either

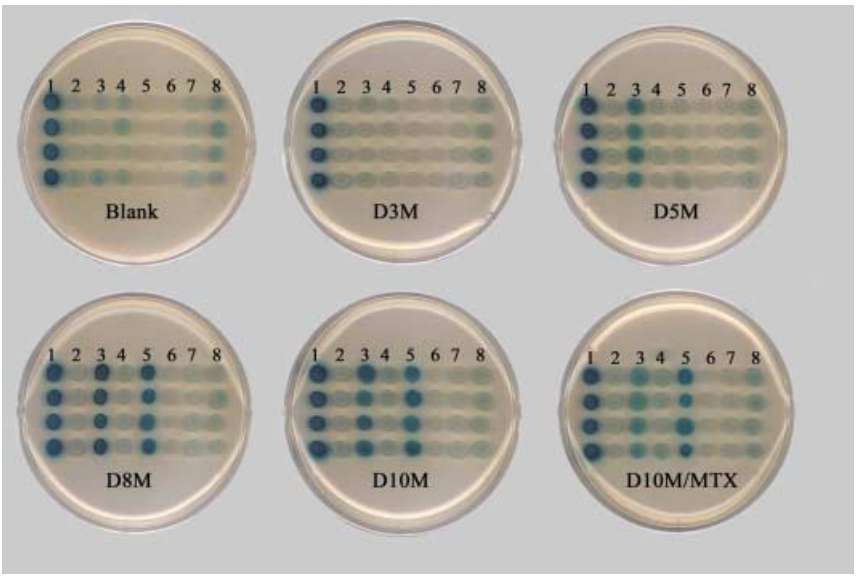


Figure 2. *X-gal plate assay of Dex – Mtx-induced* lacZ *transcription. Yeast strains containing a* lacZ *reporter gene and different LexA and/or B42 chimeras were grown on X-gal indicator plates with or without Dex – Mtx and Mtx as indicated. Columns 1 – 8 on each plate correspond to yeast strains containing different LexA- and/or B42-chimeras. 1: LexA – Sec16p, B42 – Sec16p; this is a direct protein – protein interaction used as a positive control.[27] 2: LexA, B42; here both receptors are deleted and it is used as a negative control. 3: LexA – eDHFR, B42 – $(GSG)_2$ – GR. 4: LexA – $(GSG)_2$ – mDHFR, B42 – $(GSG)_2$ – GR. 5: LexA – $(GSG)_2$ – GR, B42 – eDHFR. 6: LexA – $(GSG)_2$ – GR, B42 – $(GSG)_2$ – mDHFR. 7: LexA – eDHFR, B42. 8: LexA, B42 – $(GSG)_2$ – GR. Each plate has a different small-molecule combination, as indicated in the figure. The Dex – Mtx CIDs are at 1 μM in the plate media; Mtx, at 10 μM. The plates were incubated at 30 °C for 72 hours. GSG = Gly-Ser-Gly.*

or both receptors were deleted from the LexA and B42 two-hybrid proteins (Figure 2 and data not shown).

Surprisingly, while there was a robust signal in both the plate and liquid β-galactosidase assays with *E. coli* DHFR, there was no detectable signal with murine DHFR (Figure 2, lanes 3 and 4; Figure 3 B). With the *E. coli* DHFR, there was significant X-gal hydrolysis on plates after 72 hours at 1 μM D5M. In liquid culture, β-galactosidase synthesis began to be induced at 100 nM D5M, and at 10 μM D5M it was five times higher than the background rate. There was no detectable *lacZ* transcription above the background rate after five days in either plate or liquid assays for the murine DHFR with 10 nM to 10 μM D5M.

Transcription activation shows little dependence on CID structure

To rule out the possibility that the difference in activity between the *E. coli* and murine DHFRs was simply a reflection of different interactions with the CID, we synthesized Dex – Mtx CIDs with different chemical link-

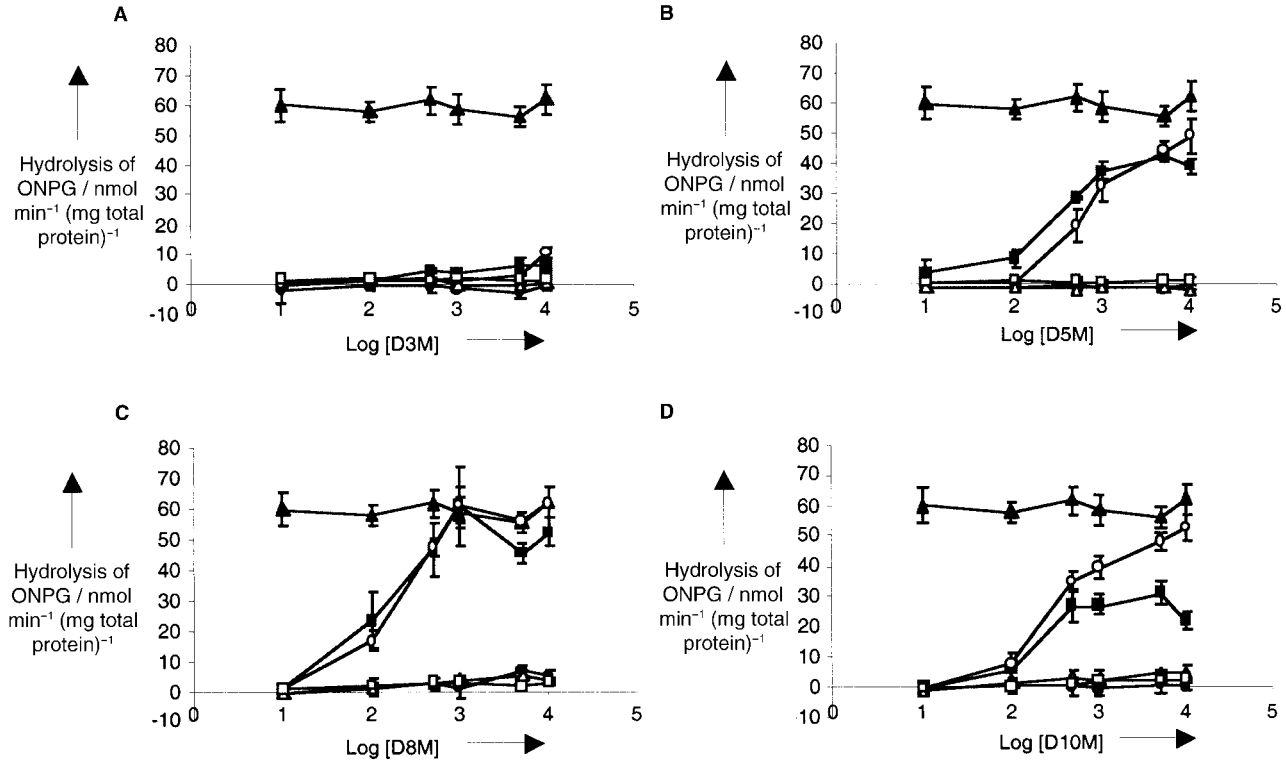


Figure 3. *More quantitative liquid assays of Dex – Mtx-induced* lacZ *transcription. Yeast strains containing a* lacZ *reporter gene and different LexA and/or B42 chimeras were grown in liquid culture for 72 hours with different concentrations of the Dex – Mtx CIDs as indicated. The rate of ONPG hydrolysis as a function of the log of CID concentration (in nM) is given for yeast strains containing different LexA and/or B42 protein chimeras. 1: LexA – Sec16p, B42 – Sec16p (▲). 2: LexA, B42 (●). 3: LexA – eDHFR, B42 – $(GSG)_2$ – GR (■). 4: LexA – $(GSG)_2$ – mDHFR, B42 – $(GSG)_2$ – GR (△). 5: LexA – $(GSG)_2$ – GR, B42 – eDHFR (○). 6: LexA-$(GSG)_2$-GR, B42-mDHFR (□). Error bars represent plus or minus the standard error obtained from at least two separate trials.*

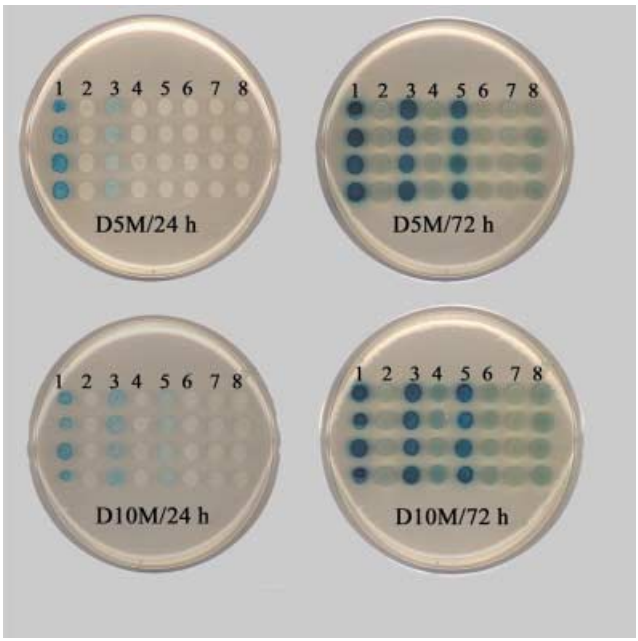


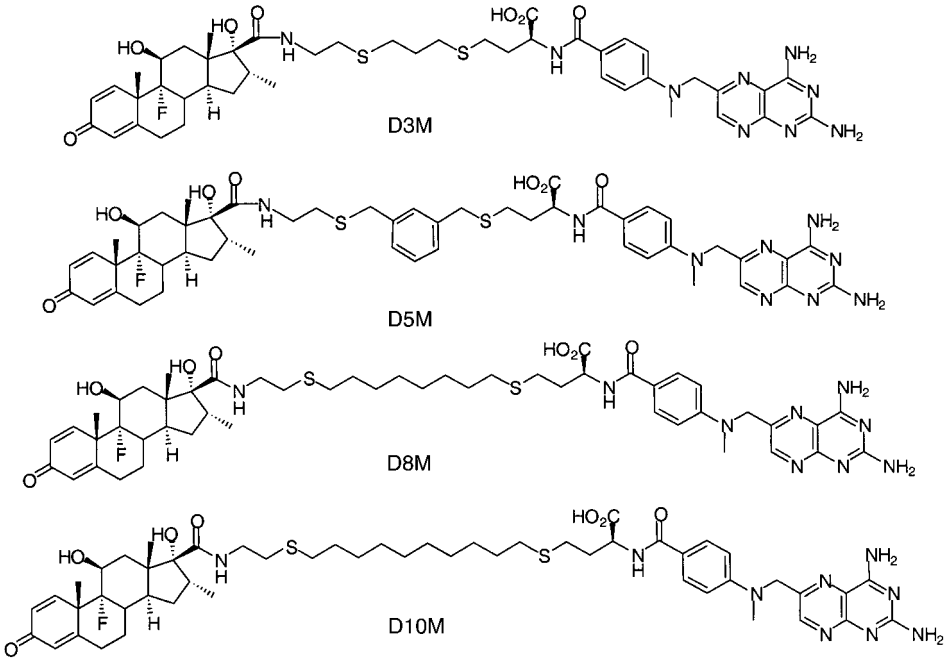
Figure 4. *A time-course experiment reveals differences in the activity of D10M and D5M. D8M behaved essentially as D10M (data not shown). X-gal plate assay of the time course of Dex – Mtx-induced* lacZ *transcription. Yeast strains containing a* lacZ *reporter gene and different LexA and/or B42 chimeras were grown on X-gal indicator plates with 10 μM D5M or 10 μM D10M, as indicated. Columns* **1** *–* **8** *are exactly as delineated for Figure 2. The plates were incubated at 30 °C for 24 or 72 hours, as indicated.*

ers (Scheme 1). Previous results with Dex – Dex small molecules have shown that hydrophobic linkers improve the cell permeability of these CIDs (V. Cornish, unpublished results). We reasoned that simple straight-chain aliphatic linkers would be least likely to disrupt the interaction between Mtx and DHFR.

The synthesis of these CIDs was facilitated by the original design of the Dex – Mtx retrosynthesis, which allows the chemical linker to be varied systematically.[1] Thiol derivatives of both Dex and Mtx were prepared and then coupled to a dihalo linker. To facilitate purification, the Dex and Mtx thiol analogues were added one at a time rather than simultaneously in a one-pot synthesis as reported previously. In the final steps, 2,4-diamino-6-bromomethylpteridine is added to complete the synthesis of the Mtx portion of the molecule, and the *tert*-butyl protecting group is removed. Under these conditions, the Dex – Mtx compounds were synthesized from two components in nine steps in 2 – 5 % overall yield. It should be noted that the synthesis of CIDs with shorter linkers, not surprisingly, was complicated by competing intramolecular reactions. In particular, the straight-chain analogue of D5M with a 5-methylene chain linker could not be prepared due to the formation of a thiacyclohexane ring when diiodo pentane was coupled to the Mtx thiol derivative.

At a gross level, the chemical linker in the CID has little effect on CID-induced transcription. With the exception of the compound with the shortest linker, D3M, all of the Dex – Mtx CIDs strongly induce transcription with the LexA – eDHFR, but not the LexA – mDHFR, constructs. These results can be seen qualitatively in the plate X-gal assays and more quantitatively in the liquid ONPG assays. On plates at a 1 or 10 μM concentration, D5M, D8M, and D10M all show strong levels of transcription activation in strains containing LexA – eDHFR and B42 – GR protein partners and weak levels or no activation at all in strains with LexA – mDHFR and B42 – GR protein partners (Figure 2). These results are confirmed over a range of Dex – Mtx concentrations in liquid culture assays (Figure 3).

Closer inspection, however, reveals subtle differences among the three CIDs that activate transcription. Based on more quantitative liquid assays, D8M consistently shows the highest absolute levels of β-galactosidase synthesis (Figure 3). Also, D8M and D10M seem less sensitive to the construction of the protein chimera than does D5M. In plate assays with the CID at 1 μM concentrations, D8M and D10M, but not D5M, induce transcription of the *lacZ* reporter gene above background levels in the LexA – GR/B42 – eDHFR strain (Figure 2). Both D8M and D10M activate transcription in the strains containing eDHFR and GR at earlier time points than does D5M at 10 μM concentrations of CID in plate *lacZ* assays (Figure 4). Also shown in Figure 4 is the fact that D10M weakly activates *lacZ* transcription in strains containing mDHFR constructs at a concentration of 10 μM, while D5M shows no such activation. Liquid as-



Scheme 1. *Dex – Mtx CIDs with different chemical linkers. Dex – Mtx CIDs with a three-methylene linker (D3M), the equivalent of a five-methylene linker (D5M), an eight-methylene linker (D8M), and a ten-methylene linker (D10M) were prepared.*

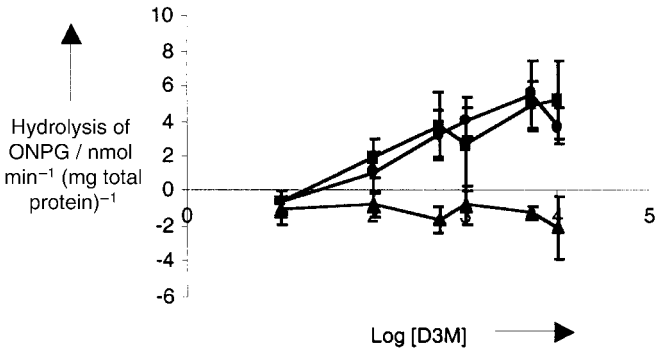


Figure 5. More quantitative liquid assays show that D8M and D10M are able to activate transcription in the LexA – $(GSG)_2$ – mDHFR/B42 – $(GSG)_2$ – GR strain. The rate of ONPG hydrolysis as a function of the log of CID concentration (in nM) for the yeast strain containing the LexA – $(GSG)_2$ – mDHFR and B42 – $(GSG)_2$ – GR protein chimeras and a lacZ *reporter gene incubated with different concentrations of D5M (▲), D8M (●), or D10M (■). Error bars represent plus or minus the standard error obtained from at least two separate trials.*

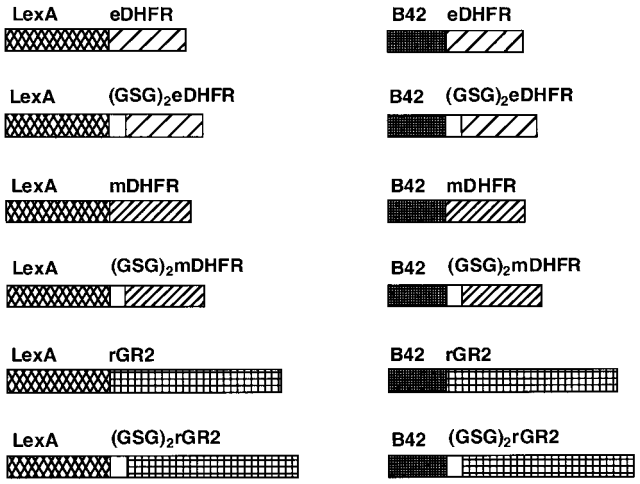


Figure 6. Protein chimeras described in this study.

says confirm that D8M and D10M are able to weakly activate transcription in the LexA – mDHFR/B42 – GR strain—no activation is observed with D5M (Figure 5).

Transcription activation depends on receptor fusion proteins

Based on both anecdotal reports and a systematic study by Estojak et al. showing that the yeast two-hybrid assay can be sensitive to the direction of the protein – protein interaction,[24] we chose to compare eDHFR and mDHFR not only with DHFR fused to LexA and GR to B42 as in our original report, but also with GR fused to LexA and DHFR to B42. In addition, we chose to engineer the protein fusions both with and without (Gly-Ser-Gly)$_2$ linkers between the receptor and two-hybrid domains since there is considerable evidence from the protein engineering literature to suggest that short amino acid linkers may be necessary to allow the two domains of a protein chimera to fold and function properly.[28] Depending on the restriction sites used, the commercial Brent two-hybrid vectors leave only a short amino acid linker between the receptors and LexA and B42 (Glu-Phe or Glu-Leu in the chimeras presented in this paper). In addition, the B42 fusion contains a 13 amino acid hemaglutanin (HA) epitope tag between the B42 sequence and the short amino acid linker.

Construction of the LexA– and B42 – receptor fusions was facilitated by the availability of commercial vectors for the Brent two-hybrid system, and plasmids encoding all 12 protein chimeras (Figure 6) were prepared by using standard molecular biology techniques. The genes encoding DHFR and GR were amplified with PCR and then subcloned into the commercial two-hybrid vectors, *pMW103* and *pMW102*, which encode LexA and B42, respectively. The (Gly-Ser-Gly)$_2$ linkers were introduced during the PCR amplification step. The regions encoding the receptors that were amplified by PCR were sequenced in full. Vectors encoding the resulting LexA– and B42 – protein chimeras were introduced in all combinations into *S. cerevisiae* strain - V248Y, a derivative of strain FY250 containing the *lacZ* reporter gene on a 2μ plasmid (*pMW106*).

In contrast to the different Dex – Mtx CIDs, there was enormous variability in the activity of the different protein chimeras. Changing the directionality of the system, that is, swapping the receptors fused to LexA and B42, had significant effects on CID-induced transcription activation. The peptide linker turned out to have little effect on activity, and so this data is omitted for simplicity. While D5M, D8M, and D10M cannot be distinguished in plate or liquid assays at 10 μM concentrations after more than 3 days of incubation with the CID, earlier time points and lower concentrations reveal greater levels of β-galactosidase synthesis for the LexA – eDHFR/B42 – GR combination than for the LexA – GR/B42 – eDHFR combination (Figures 2 and 4). This difference is most pronounced in the case of D5M. The levels of X-gal hydrolysis in the mDHFR strains are too close to background levels to draw conclusions from the plate assays (Figure 2). In the more quantitative liquid assays, however, both D8M and D10M show induction of β-galactosidase clearly above experimental error in the LexA – mDHFR/B42 – GR strain (Figure 5); whereas there is no detectable activation when the orientation is swapped to LexA – GR/B42 – mDHFR (data not shown).

Differences in transcription activation are not due to variations in protein expression levels

The first question we posed was whether or not the differences in transcription activation simply reflected variations in protein expression levels. Decreasing the concentration of the DBD or AD chimeras could decrease the amount of reconstituted transcriptional activator in the cell, or there could be a complex relationship between protein expression levels and transcription activation. Thus, we compared the levels of expression of the DHFR and GR chimeras by Western blot analysis (Figure 7). Briefly, yeast strains containing the different LexA and B42 chimeras were grown to mid-log phase, the cells were lysed, and the soluble and insoluble fractions were combined. Total protein concentrations were determined by Bradford assays with bovine serum albumin (BSA) as a standard. Each sample was normalized such that 2 μg of total protein was analyzed. The protein mixture

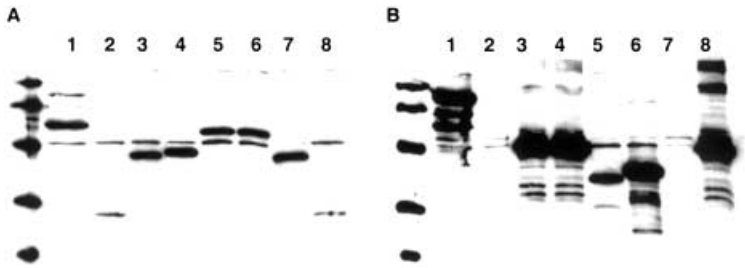


Figure 7. *There are no significant variations in the expression levels of either the LexA or the B42 protein chimeras. A Western blot of a 12% SDS-PAGE gel of lysed yeast cells grown to mid-log phase and containing different LexA and/or B42 fusion proteins. Columns 1 – 8 are exactly as delineated for Figure 2. Biotinylated protein M_W markers (21.5 kD, 31 kD, 45 kD, 66.2 kD, and 97.4 kD) were run in the far left lane of each gel. Gel A was stained with an anti-LexA antibody, gel B, with an anti-hemaglutanin (HA) antibody that recognizes an HA epitope tag engineered into the B42 protein fusions. Both gels were incubated with an avidin – horse radish peroxidase fusion to detect the M_W markers. The LexA and B42 fusion proteins show the expected molecular weight. LexA and B42 alone are not observed.*

was separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and then stained with either an anti-LexA monoclonal antibody for the LexA fusion proteins or an anti-HA monoclonal antibody that recognized an HA epitope engineered into the B42 fusion proteins. A comparison of all 16 strains examined showed little variation in the expression levels of either the LexA or B42 fusion proteins. The minor fluctuations observed have no correlation with the *lacZ* transcription data. A representative subset of this data is shown in Figure 7. For the B42 fusion proteins, truncated products are observed; these products, however, are consistent for the eDHFR and mDHFR fusion proteins, which suggests that they do not account for the difference in transcription activation observed for these two proteins.

Differences in transcription activation are not due to disruption of Mtx binding to DHFR fusion proteins

An in vivo activity assay was then used to ensure that the DHFR chimeras were being expressed in an active form in the yeast cells. Yeast strains containing the different LexA and B42 chimeras were grown to mid-log phase in selective media, and fluorescein-tagged methotrexate was added to the solution for binding to DHFR. After a 24 hour incubation period, the cells were washed and transferred to fresh media to allow efflux of unbound methotrexate. The cells were then lysed and fluorescence of the lysate was analyzed. Fluorescence values were normalized by determining total protein concentrations with a Bradford assay. The amount of fluorescence in all strains containing the DHFR fusion proteins is comparable (Figure 8), which indicates that both the *E. coli* and murine DHFRs are being expressed in an active form in vivo.

Differences in transcription activation are not due to disruption of DNA binding in LexA – receptor fusion proteins

Having shown that the DHFR domain was being expressed in an active form in vivo, the remaining question was whether or not the DNA-binding activity of the LexA domain was affected in the LexA – DHFR fusion proteins. An in vivo transcription repression assay has been established for verifying the integrity of LexA fusion proteins. This assay measures the levels of transcription of a *lacZ* gene under control of the *GAL1* promoter. Two tandem LexA operators have been engineered between the *GAL1* promoter and the *lacZ* gene such that binding of functional LexA or LexA fusion proteins disrupts transcription of the *lacZ* gene. We compared the levels of *lacZ* transcription in yeast cells containing the *lacZ* reporter gene in the absence of LexA, in the presence of LexA, and in the presence of the LexA – eDHFR, LexA – mDHFR, or LexA-GR fusion proteins (Figure 9). As shown in Figure 9, with no LexA, the *GAL1* promoter activated transcription of the *lacZ* gene. With LexA alone, transcription is diminished, but not completely repressed. These low levels of transcription with LexA alone have been attributed to the LexA protein itself

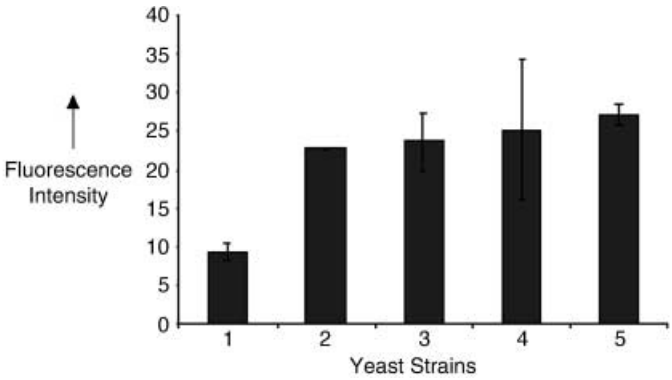


Figure 8. *An in vivo fluorescence assay indicates that both the* E. coli *and murine DHFRs are expressed in an active form in vivo. Yeast strains containing the various DHFR fusion proteins were incubated in selective media with an excess of fMtx. Unbound fMtx was then allowed to diffuse out of the cells through successive media transfers. The cells were finally lysed, and fluorescence intensity, indicative of DHFR activity, was measured for each of the yeast strains. 1: LexA, B42. 2: LexA – eDHFR, B42 – $(GSG)_2$ – GR. 3: LexA – $(GSG)_2$ – mDHFR, B42 – $(GSG)_2$ – GR. 4: B42 – eDHFR, LexA – $(GSG)_2$ – GR. 5: B42 – $(GSG)_2$ – mDHFR, LexA – $(GSG)_2$ – GR. Error bars represent plus or minus the standard error obtained from at least two separate trials.*

being slightly activating.[29] *LacZ* transcription was completely repressed in the presence of the LexA – eDHFR, LexA – mDHFR, or LexA – GR fusion protein. Thus, the repression assay provides further evidence that both the *E. coli* and murine DHFR – LexA chimeras are able to fold and function properly in vivo.

Discussion

This work provides the first systematic characterization of a small-molecule three-hybrid system. These systems show promise for screening pools of proteins or small molecules based on ligand – receptor interactions. The inherent assumption is that the small molecules and protein chimeras can be varied without disrupting the transcription read-out. Yet, there are anecdotal

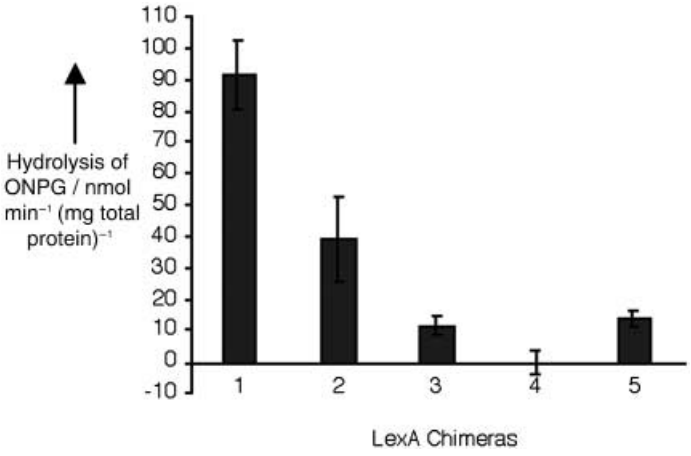


Figure 9. *Repression assays show that all of the LexA chimeras are able to fold and bind DNA properly. In these assays, a reporter gene with two tandem LexA operators between the* GAL1 *promoter and the* lacZ *gene is used such that functional LexA fusion proteins disrupt transcription of the* lacZ *reporter.* β*-Galactosidase activity levels of different yeast strains containing* pJK101 *with and without different LexA chimeras were determined in liquid culture with ONPG. 1:* pJK101 *is used without any LexA chimeras to show full activation of the* lacZ *gene. 2:* pJK101 *with LexA. 3:* pJK101 *with LexA – eDHFR. 4:* pJK101 *with LexA – $(GSG)_2$ – mDHFR. 5:* pJK101 *with LexA – $(GSG)_2$ – GR. LexA – $(GSG)_2$ – mDHFR exhibited the highest repression of the* lacZ *gene; thus, its value of ONPG hydrolysis was subtracted from all other repression measurements. Error bars represent plus or minus the standard error obtained from at least twelve separate trials.*

reports of high-affinity ligand – receptor pairs that cannot be detected with this assay. We sought to carry out a systematic study of our Dex – Mtx three-hybrid system that would directly test the influence of the ligand – receptor pair on the levels of transcription activation.

The most intriguing result is that, though it is well established that both eDHFR and mDHFR bind Mtx with affinity in the low pM range, only the eDHFR – Mtx interaction activates transcription significantly in this system. This result provides a clear-cut example of related high-affinity small-molecule – protein interactions that give different levels of transcription activation in the yeast three-hybrid system. Since the *E. coli* DHFR produces a strong signal, it can be reasoned that the Dex – Mtx CID is cell permeable and that the B42 – GR/Dex – Mtx/DHFR – LexA ternary complex is in an orientation productive for transcription activation.

Our initial efforts to understand the basis for the difference in activity between the two receptors show that the LexA – DHFR fusion proteins are all being expressed in an active form in vivo, thereby ruling out a trivial explanation for the receptor dependence. Western blot analysis shows that the expression levels of the *E. coli* and murine DHFR fusion proteins are all quite similar, and in vivo activity assays, both for Mtx binding by DHFR and for DNA binding by LexA, establish that both halves of the LexA – DHFR fusion proteins remain active.

In addition, a comparison of the levels of transcription activation by using CIDs with different chemical linkers and different protein chimeras shows that the system is much more sensitive to the nature of the protein chimera than the CID. This result has the obvious practical implication that efforts to detect a new ligand – receptor interaction should focus on variations in the protein chimeras, rather than the CID. Further biochemical characterization is needed to determine whether the variations in transcription levels result simply from differences in stability of the ternary complex or have more subtle implications for the mechanism by which transcriptional activators interact with the transcription machinery.

Perhaps the simplest explanation for the *lacZ* transcription results is that while eDHFR and mDHFR have similar affinity for Mtx, the stability of the GR/Dex – Mtx/DHFR ternary complexes differs for the two proteins. Currently, we are carrying out in vitro biochemical studies to determine the stability of the GR/Dex – Mtx/DHFR ternary complexes for the different protein chimera and CID combinations reported here to test this hypothesis. In addition, mutants of both the eDHFR and mDHFR fusion proteins that alter the NADPH dependency are being tested in the transcription assay. Given the particularly high affinity of the Mtx – DHFR interaction, it is interesting to speculate that the transcription levels will not be solely due to a thermodynamic effect. Both proteins bind to Mtx in a two-step mechanism, with isomerization to the second, high-affinity complex being the rate-determining step. The initial complex for mDHFR is almost 100 times less stable than for eDHFR and is dominated by a rapid off rate (k_{off}). The transcriptional activator may need to be bound to DNA with a minimum half-life in order for Mediator to bind and stabilize the RNA Pol II preinitiation complex, or the half-life of the Mtx – DHFR interaction may influence the lifetime of the TATA-binding-protein – promoter complex during successive rounds of transcription.

The three-hybrid assay should provide a powerful method for studying small-molecule – protein interactions. One ligand – receptor pair (one half of the CID) can be held constant and used as an anchor, while the other ligand – receptor pair is varied. Because the small-molecule – protein interaction controls transcription of a reporter gene in vivo, the assay can be run as a growth selection allowing millions of protein or small-molecule variants to be tested simultaneously. The anchor ligand can be linked to a small-molecule drug and used to screen a cDNA library to find the cellular target for that drug. The system can be used in the design of a new high-affinity ligand – receptor pair or to study a natural ligand – receptor interaction. For this assay to be general, however, it must be able to detect small-molecule – protein interactions with a range of structures and dissociation constants. Despite this potential generality, little has been done to characterize the influence of the small-molecule – protein interaction on the transcription read-out. Here we have carried out a systematic study on the activity of a bacterial and a mammalian DHFR homologue in the yeast three-hybrid assay. Surprisingly, we find that, though both proteins are inhibited by Mtx with picomolar affinity, the transcription read-out for the two differs dramatically. This work lays the groundwork for further biochemical studies to understand how the ligand – receptor interaction determines the strength of the transcription read-out. These studies should not only allow us to improve the generality of the three-hybrid assay, but should also add to our understanding of the mechanism of transcription activation in eukaryotes.

Experimental Section

Chemical synthesis: A complete description of the synthesis of the Dex – Mtx CIDs is given in the Supporting Information, together with full experimental details and compound characterization.

Biological methods: A more complete description of the general methods for molecular biology, construction of the LexA and B42 fusion proteins, preparation of the yeast strains, and Western blot analysis is given in the Supporting Information.

Plate *lacZ* transcription assays: *LacZ* transcription assays were carried out on plates essentially as previously described.[27] All yeast strains were stored as 20% glycerol stocks in 96-well plates at −80 °C. For the plate assay, the yeast strains were first transferred from a 96-well plate glycerol stock into 96-well plates with synthetic complete (SC) media, containing 2% glucose but lacking uracil, histidine, and tryptophan, and then incubated at 30 °C with agitation (80 revolutions per minute (rpm)) for 3 days. The yeast strains were then transferred from the 96-well liquid culture plates onto X-gal indicator plates, with or without the small molecules Dex – Mtx, Mtx, and/or Dex and lacking the appropriate selective nutrients, and incubated at 30 °C for several days. The small molecules were simply added together with the nutrients when making the X-gal indicator plates.

Liquid *lacZ* transcription assays: Liquid assays with ONPG were performed in Corning Costar 96-well plates by using an Invitrogen (www.invitrogen.com) protocol for detection of β-galactosidase activity in microtiter plates. Yeast strains were first transferred from 20% glycerol stocks into 96-well plates with SC media, containing 2% glucose but lacking uracil, histidine, and tryptophan, and then incubated at 30 °C with agitation (80 rpm) for 3 days. At this time, yeast strains were transferred to 96-well plates with SC media, containing 2% galactose and 2% raffinose but lacking uracil, histidine, and tryptophan, with and without varying concentrations of Dex – Mtx, Dex, and/or Mtx compounds. These plates were placed at 30 °C with agitation (80 rpm) for 3 days.

Yeast cells were washed with phosphate buffered saline (PBS, pH 7.4) and lysed in 0.25 M tris(hydroxylmethyl)aminomethane (Tris, pH 8.0) by repeated freeze – thaw cycles. Half of the cell lysate was used to measure the absolute β-galactosidase activity of the cells by incubation with ONPG (1 mg mL^{-1}) for 30 min at 37 °C, after which time the reaction was stopped by adding 1 M sodium carbonate, and the absorbance of the solution at 420 nm was measured by using the HTS 7000 Plus BioAssay plate reader. The other half of the cell lysate solution was used to calculate the total protein concentration of the lysate by using the Bradford method with BSA as the standard. The rates of ONPG hydrolysis were normalized to the total protein concentration.

In vivo assay for Mtx binding by DHFR: Yeast strains were grown in SC media (1 mL), containing 2% glucose but lacking uracil, histidine, and tryptophan, at 30 °C with agitation (100 rpm) for 3 days. A portion of the glucose cultures was then used to inoculate SC media (5 mL), containing 2% galactose and 2% raffinose but lacking uracil, histidine, and tryptophan. The culture was grown at 30 °C with agitation (100 rpm) for 2 days. Fluorescein-tagged Mtx (fMtx, Molecular Probes) was added to a final concentration of 10 μM, and the culture was grown for another 22 h. The cells were then washed with synthetic defined (SD) media, transferred to SC media (5 mL), containing 2% galactose and 2% raffinose but lacking uracil, histidine, tryptophan and fMtx, and grown at 30 °C with agitation for 45 min. The wash, media transfer, and 45 min growth cycle were repeated. Cells were finally washed and lysed in lysis buffer (1.5 mL; 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.5, 10% glycerol, 0.5% Triton X-100, 150 mM NaCl, 0.771 mg mL^{-1} of dithiothreitol, 16.7 μL mL^{-1} of protease inhibitor, 20 μL mL^{-1} of octanol) by repeated freeze – thaw cycles. Fluorescence in the lysate was measured using a fluorescence spectrometer (ISA Fluoromax-2; excitation and emission wavelengths were 490 nm and 516 nm, respectively). A Bradford assay was used to determine the total concentration of protein in the lysate, so that the fluorescence values of the samples could be normalized.

In vivo assay for LexA DNA-binding activity: The LexA repression assays were carried out by using X-gal plate and ONPG liquid assays essentially as for the CID-induced transcription assays. The only difference is that the LexA fusion proteins were introduced into yeast strain FY250 containing the reporter plasmid *pJK101*. *pJK101* was purchased from Origene and has two tandem LexA operators inserted between the *lac*Z gene and the *GAL1* promoter.

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