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A Conditionally Expressed Third Partner Stabilizes or Prevents the Formation of a Transcriptional Activator in a Three-hybrid System*

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We describe a three-hybrid system that involves three polypeptides that allow or prevent the formation of the transcriptional activator. Beside the two-hybrid fusion proteins, the third partner is under the control of the Met25 promoter, which is positively regulated in medium lacking methionine. We document a situation where such a third partner promotes interaction between two proteins, one fused to a DNA-binding domain and the other fused to an activator domain. This is demonstrated for cdk7-MAT1 interaction stabilized by the presence of cyclin H; these three polypeptides are found either free or associated with the transcription/DNA repair factor TFIIH. We also document the capacity of our system to conditionally inhibit the interaction between two polypeptides that otherwise elicit a positive two-hybrid response. This is demonstrated for Ras-Raf interaction precluded by an excess of Raf. The presence of a methionine-regulated promoter provides an "on" or "off" switch for the formation of the transcriptional activator, thus also providing an excellent control to evaluate the activation or inhibition properties of the third partner.

Molecular events are usually coordinated by protein-protein interactions that can induce protein modifications (*e.g.* phospho-dephosphorylation, alkylation, glycosylation, proteolytic cleavage, etc.), eventually driving conformational changes affecting the biological activity of the protein complex. To detect protein-protein interactions, several techniques such as affinity chromatography, coimmunoprecipitation, and glycerol gradient sedimentation are currently used. Following the work of Ptashne's group (1), a new technique called the yeast two-hybrid system based on the reconstitution of a transcriptional activator complex was established (2). This technique detects interactions between two fused proteins that contain in addition to their own sequence a DNA-binding domain (DBD)[1] or an activator domain. Examples of studies using the yeast two-hybrid system include the detection of the cell cycle factors Cip1 (3) and cyclin H (4); the identification of proteins involved in apoptosis (5); the interaction study of the repair complexes involving either XPA and ERCC1 (6) or MLH1, MSH2, and PMS1 (7); and the study of interactions between the signal transduction factors Ras and Raf (8, 9). Furthermore, this technique helped us to understand the architecture and to determine the interactions between the various subunits of TFIIH, a transcription factor also involved in DNA repair (10).[2]

Although the two-hybrid system was shown to be successful in detecting interaction between two well defined proteins, this method fails to detect most of contacts between proteins that are part of a larger protein complex such as the RNA polymerase II holoenzyme and the TFIIH or the TFIID complexes. Indeed, each polypeptide, when out of its native complex, could be targeted nonspecifically by other proteins that contact the protein area usually covered by the partner subunits of the complex (*e.g.* for TFIIH, there are nine putative interacting polypeptides) (12). On the other hand, one polypeptide belonging to such a larger complex may not be sufficient to trap another polypeptide due to the weakness of the interaction; detection of a target protein would thus require multipoint attachment through more than one polypeptide.

In the present study, we describe a novel three-hybrid system that allows the use of two proteins as a bait to screen available cDNA libraries to detect a third partner. Such a system allows the detection of ternary complex formation as well as inhibitors preventing interaction between the two previously defined fused protein. The advantage of the present three-hybrid system compared with the existing ones (13, 14), is the possibility of controlling the expression of the third partner cloned under a conditional promoter such as the methionine-repressed Met25 promoter (15, 16).

To illustrate our three-hybrid system, we used the property of three polypeptides, cdk7, cyclin H, and MAT1 (17–19), to form a biologically active Cdk-activating kinase (CAK) complex. This complex, which exists free in the cell, is also found as part of TFIIH, a multiprotein complex essential for DNA transcription and DNA repair as well as for cell cycle regulation (20). We show here how each of the three partners interacts with each other. Furthermore, the three-hybrid system presents an interesting approach to identify proteins that can inhibit interaction between two proteins of interest. In this case, we used the interaction properties of the two oncogenes Ras and Raf, which function in the transduction of signals to control cell growth and differentiation (9). Addition of Raf protein as a competitor inhibits the formation of the transcriptional activator complex Gal4DBD-Ras/Gal4AD-Raf.

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[1] The abbreviations used are: DBD, DNA-binding domain; CAK, Cdk-activating kinase; HA, hemagglutinin; ORF, open reading frame; ADH, alcohol dehydrogenase.

[2] V. Moncollin, S. Humbert, C. Malaguti, A. Fery, J. R. Hwang, A. Poterszman, D. Moras, and J. M. Egly, submitted for publication.

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MATERIALS AND METHODS

Yeast Strains and Manipulations—We used the L40 strain (*MAT***a**, *trp1, his3, leu2, ade2, LYS2::$(LexAop)_4$-HIS3, URA3::$(LexAop)_8$-lacZ*) for ternary complex analysis (9) and the HF7c (*ura3, his3, lys2, ade2, trp1, leu2, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, URA3::$GAL4_{17mers(x3)}$-$CyC1_{TATA}$-lacZ*) strain (21) for inhibition analysis. Composition of media, yeast growth, transformation, and selection were conducted as described (22). Expression from the Met25 promoter is repressed in the presence of 1 mM methionine, although this tight control displays some leakiness when the Met25 promoter is on a multicopy plasmid (16).

Plasmids Constructions—A methionine-regulated expression cassette was inserted at the unique *Pvu*II site of plasmid pGBT9 (23), pGBT10 (24), pHP5,[3] pBTM116 (9), and pVJL10 (25), giving plasmids pGBT9-3H, pGBT10-3H, pGBT11-3H, pLex9-3H and pLe × 10-3H, respectively. In pGBT9-3H/B and pGBT10-3H/B, a *Bgl*II site replaces a *Srf*1 site. This methionine-regulated expression cassette contains region −474 to −1 of the Met25 promoter (15) followed by the sequence 5′-ATGGGCCATATGGCTTCTAGCTATCCTTATGACGTGCCTGACTATGCCAGCCTGGGAGGACCTTCTAGTCCTAAGAAGAAGAGAAAGGTGGCGGCCGCATTAGCCCGGGCTGATCTCCCATGTCTCTACTGGTGGTGGTGCTTCTTTGGAATTATTGGAAGGTAAGGAATTGCCAGGTGTTGCTTTCTTATCCGAAAAGAAATAAATTGAATTGAATTGAAATCGA-3′ (*Not*I and *Srf*1 sites are underlined), which encodes peptide MGHMASSYPYDVPDYASLGGPSSPKKKRKVAAALARADLPCLYWWWCFFGIIGR-stop (Fig. 1). In variants 3H/B, the sequence is 5′-ATGGGCCATATGGCTTCTAGCTATCCTTATGACGTGCCTGACTATGCCAGCCTGGGAGGACCTTCTAGTCCTAAGAAGAAGAGAAAGGTGGCGGCCGCATTAGCCCGAAGATCTTCGGGCTGA-3′ (*Not*I and *Bgl*II sites are underlined) and encodes peptide MGHMASSYPYDVPDYASLGGPSSPKKKRKVAAALARRSSG-stop. In the sequences of both peptides, the hemagglutinin (HA) epitope is underlined, and the nuclear localization sequence is double underlined. Downstream from these sequences, a region of the phosphoglycerate kinase transcription terminator was inserted corresponding to the *Bgl*II-*Hin*dIII terminator fragment from plasmid pEMBLYe30/2 (26).

For ternary complex study, all the templates used for the polymerase chain reactions were constructions previously made in our laboratory (17). The open reading frames (ORFs) coding for cdk7 and cyclin H were introduced in pLex9-3H as fusion proteins with LexA, downstream of the ADH promoter, or with the HA epitope, downstream of the Met25 promoter, respectively. The resulting plasmid is referred to as [pLex9-cdk7/Met-cyclin H]. The cdk7 fragment was obtained by polymerase chain reaction with the sense primer including an *Eco*RI cloning site (underlined) 5′-AGTCGTGAATTCATGGCTCTGGACCTGAAG-3′ and antisense primer also including an *Eco*RI cloning site 5′-GATCGTGAATTCTTAAAAAATTAGTTTCTTGGGCAA-3′. The amplified fragment was cloned into the *Eco*RI site of pLex9-3H (Fig. 1). The cyclin H fragment was obtained by polymerase chain reaction using sense primer including a *Not*I site 5′-GATCGTGCGGCCGCAATGTACCACAACAGT-3′ and an antisense primer 5′-GATCGTGCGGCCGCTTAGAGAGATTCTACCAG-3′. The amplified fragment was cloned into the *Not*I site of pLex9-3H. The ORF encoding MAT1 was also obtained by polymerase chain reaction and inserted in the *Eco*RI site of pVP16, in fusion with the activator domain of VP16. The sense primer including an *Eco*RI site had the sequence 5′-GATCAGGAATTCCCATGGAGGATCAGGGTT-3′, and the antisense primer had the sequence 5′-GATCAGGAATTCTTAACTGGGCTGCCAGAA-3′.

For the inhibition study, we have constructed pGBT9-3H/B-derived plasmids as follows. In [pGBT-Ras/Met-0], the H-Ras (V12) ORF (*Eco*RI-*Sal*I fragment of pBTM116-Ras (9)) was cloned in frame downstream of the GAL4 DNA-binding domain in pGBT9-3H/B. The plasmid [pGBT-Ras/Met-Raf] was obtained by adding at the 5′- and 3′-ends of the *Eco*RI-*Bam*HI cRaf-1 fragment from pGAD-Raf (27), a linker containing a *Not*I site, and by cloning the resulting fragment into the *Not*I site of the Met25 cassette of [pGBT-Ras/Met-0] (Fig. 1). All in frame fusions were checked by sequencing.

RESULTS

Several vectors derived from already well characterized two-hybrid plasmids were constructed. Vector pLex9-3H, elaborated from pBTM116 (17), expresses the LexA DBD under the control of the ADH promoter and contains the TRP1 selection gene, allowing its selection on a tryptophan-lacking medium. Vector pGBT9-3H/B, derived from pGBT9 (23), expresses the GAL4 DBD under the control of the ADH promoter and contains the TRP1 selection gene. Each vector possesses an expression cassette including the Met25-repressible promoter (pMet25), multiple cloning sites, and a phosphoglycerate kinase terminator (Fig. 1). These plasmids allow the constitutive expression of the protein fused to the DBD as well as the conditional expression of another polypeptide under the control of the Met25 promoter (expressed only in medium lacking methionine).

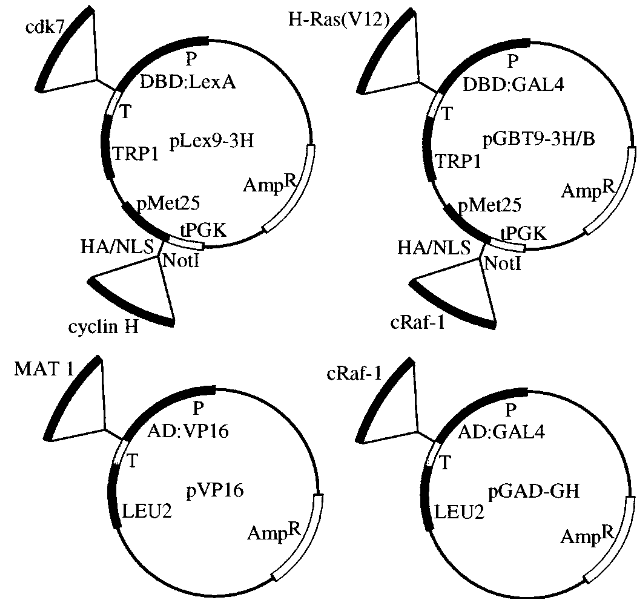

FIG. 1. **Three-hybrid system vectors.** In addition to the TRP1 selection gene, pLex9-3H carries the LexA DBD and pGBT9-3H/B the GAL4 DBD; both of them are cloned between the promoter (*P*) and terminator (*T*) of the alcohol dehydrogenase gene (*ADH*). These two plasmids possess a methionine-regulated Met25 promoter (pMet25), cloning sites, a HA epitope, a nuclear localization signal (*NLS*), and a phosphoglycerate kinase terminator. cdk7 ORF is cloned in fusion with the LexA sequence, and the cyclin H ORF is cloned in the *Not*I site under the control of the Met25 promoter to result in the [pLex9-cdk7/Met-cyclin H]. H-Ras (V12) ORF is cloned in fusion with the GAL4 DBD sequence of the pGBT9-3H/B, and cRaf-1 ORF is cloned in the *Not*I site under the control of pMet25 to result in [pGBT-Ras/Met-Raf]. Schematic maps of plasmids MAT1pVP16 and cRaf-1pGAD are also shown.

Formation of a Ternary CAK Complex—We and others have already evidenced the existence of a protein complex containing in addition to the cdk7 kinase and the cyclin H, a third protein, MAT1 (Refs. 17–19; Fig. 2*C*, *lane 13*). Moreover, using the previously established two-hybrid system, we were able to detect an interaction between cdk7 and cyclin H (10, 28). Although it was demonstrated that MAT1 strongly stimulates the phosphorylation by the cdk7-cyclin H complex of a synthetic ctd substrate, which mimics the carboxyl-terminal domain of the largest subunit of RNA polymerase II, we failed to provide evidence demonstrating any interactions in a two-hybrid system between MAT1 and either cdk7 or cyclin H. In the present study, to demonstrate the reconstitution of a full CAK complex in yeast, we used the pLex9-3H plasmid and the pVP16 vector, which contains the VP16 activation domain under the control of an ADH promoter and the LEU2 selection gene, allowing its selection on a leucine-lacking medium. In our three-hybrid system, MAT1 is expressed fused to the VP16 transcription activator motif in the pVP16 plasmid (Fig. 1), whereas cdk7 and cyclin H are both introduced in pLex9-3H. The resulting [pLex9-cdk7/Met-cyclin H] plasmid contains cdk7 in fusion with the DNA-binding motif of LexA and cyclin H under the control of the Met25 promoter.

L40 yeast cells cotransformed with [pLex9-cdk7/Met-cyclin

[3] H. P. Xu and J. Camonis, unpublished data.

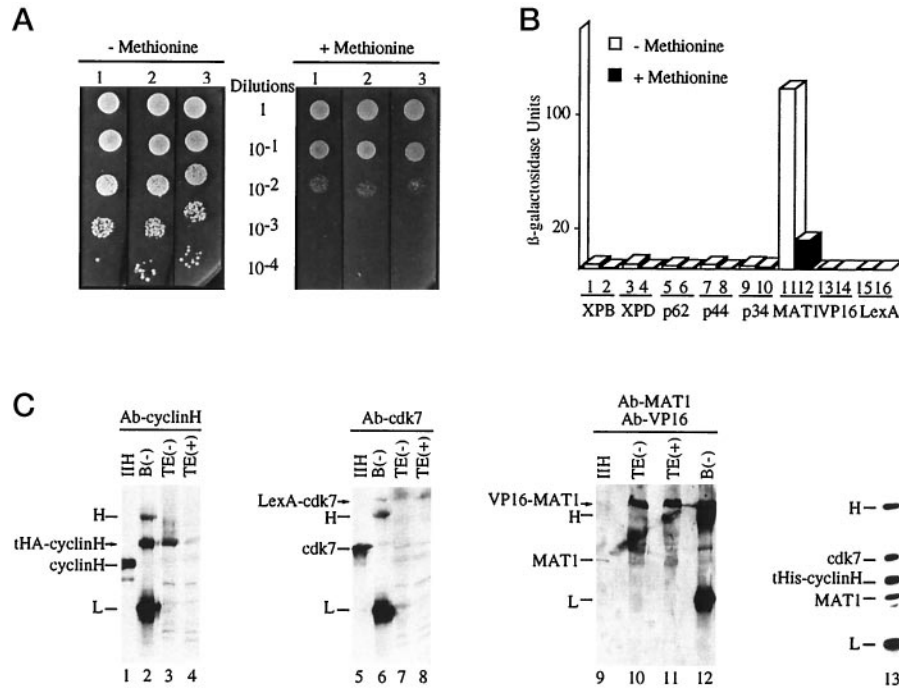

FIG. 2. *A*, three different clones of L40 yeast cells cotransformed with [pLex9-cdk7/Met-cyclin H] and MAT1pVP16 were spotted at various dilutions, as indicated at the *center* of the panel, on a minimal selective medium lacking histidine. Yeast growth was performed during 4 days at 30 °C, in the absence (−) or the presence (+) of 1 mM methionine, controlling the expression of the third partner, cyclin H. *B*, β-galactosidase assay on yeast cell extracts cotransformed by [pLex9-cdk7/Met-cyclin H] and TFIIH subunits XPB, XPD, p62, p44, p34, or MAT1 cloned in pVP16 (*lanes 1–12*); [pLex9-cdk7/Met-cyclin H] and pVP16 without any fusion (*lanes 13* and *14*); or [pLex-0/Met-cyclin H], lacking cdk7, and MAT1pVP16 (*lanes 15* and *16*). The permeabilized cell assays (21) were performed on yeast grown at A_{600} = 1 in 20 ml of a minimal selective medium containing (+) or lacking (−) methionine. *C*, Western blot on total yeast extract. Cells were grown in the presence (*TE(+)*, *lanes 4*, *8*, and *11*) or the absence (*TE(−)*, *lane 3*, *7*, and *10*) of 1 mM methionine. *IIH*, purified TFIIH; heparine high pressure liquid chromatography fraction. Immunoprecipitation on a total yeast extract using anti-HA antibodies (*B(−)*) from cells grown on a methionine-lacking medium. The Western blots were revealed with antibodies (*Ab*) as indicated at the *top* of each panel. *Lane 13* shows an immunoprecipitation with anti-cdk7 antibodies of the CAK complex expressed in baculovirus-infected cells and previously purified on a nickel chelated affinity column using the hisidine tag of the cyclin H. *L*, antibody light chain. *H*, antibody heavy chain.

H] and MAT1pVP16 vectors were grown in the absence or in the presence of 1 mM methionine. On a minimal medium lacking leucine and tryptophan to select both plasmids and lacking methionine to permit expression from the Met25 promoter, [pLex9-cdk7/Met-cyclin H]-MAT1pVP16-transformed cells were observed to grow in the absence of histidine for dilutions ranging from 1 to 10^{-4} (Fig. 2*A*, *left panel*). In contrast, inhibition of cyclin H expression by growing in presence of 1 mM methionine allowed colony growth in the absence of histidine, only for the lowest dilutions (Fig. 2*A*, *right panel*). To control whether the expression of the cyclin H could influence the expression of the two fusion proteins (VP16-MAT1 and LexA-cdk7), we performed Western blotting on yeast total extracts (Fig. 2*C*). Whether the cyclin H is expressed (*TE(−)*) or not (*TE(+)*), no differences in the expression levels of LexA-cdk7 or VP16-MAT1 were observed (Fig. 2*C*, compare *lanes 7* and *10* with *lanes 8* and *11*), demonstrating that the expression of cyclin H does not affect expression levels of cdk7 or MAT1. These results demonstrate that the expression of the third polypeptide, cyclin H, leads to the formation of a protein complex between the three overexpressed polypeptides cdk7, MAT1, and cyclin H. Such complex was already evidenced by coinfecting insect cells with baculoviruses containing the cdk7, cyclin H, or MAT1 cDNA (Ref. 10; Fig. 2*C*, *lane 13*). In addition, a much higher production of the CAK complex than the cdk7/MAT1 complex was always obtained upon infection with the corresponding baculoviruses (data not shown).

To further demonstrate that the reporter gene transcriptional activation resulted from the expression of the cyclin H protein as a third partner, we used a β-galactosidase assay. When grown in the absence of methionine, [pLex9-cdk7/Met-cyclin H]-MAT1pVP16-transformed yeasts demonstrated approximately a 7-fold increase in the enzyme activity compared with transformants grown in the presence of methionine (Fig. 2*B*, compare *lanes 11* and *12*). As controls, transformed cells with the [pLex9-cdk7/Met-cyclin H] vector in addition to pVP16 vector expressing only the VP16 activation domain and lacking the MAT1 fusion part, or transformed cells with the [pLex9–0/Met-cyclin H] without the cdk7 sequence in addition to MAT1pVP16 vector did not show a notable β-galactosidase activity at any methionine concentration examined (Fig. 2*B*, see *lanes 13–16*). The weak but significant β-galactosidase activity observed in the absence of cyclin H expression (Fig. 2*B*, *lane 12*) likely results from some weak association between cdk7 and MAT1, as previously deduced from structural studies (28) and immunoprecipitation on extracts of insect cells coinfected with baculoviruses coding for cdk7 and MAT1 (data not shown). Nevertheless, under our experimental conditions, the Met25 promoter is not totally repressed. As a further control to demonstrate the specificity of the ternary complex formation, we did the following experiment. When either one of the five core TFIIH subunits (12), XPB, XPD, p62, p44, or p34, was fused to VP16 instead of MAT1 and used to transform the yeast strain together with [pLex9-cdk7/Met-cyclin H], no interaction was observed (Fig. 2*B*, *lanes 1–10*), thus demonstrating the specificity of the ternary complex formation inside the holo-TFIIH complex.

Taken together, our results demonstrate that the activation of the reporter genes transcription by the reconstituted activator LexA/VP16 results from the formation of a LexA-cdk7-VP16-MAT1 protein complex promoted by a third partner, cyclin H. Such protein complex can be visualized by

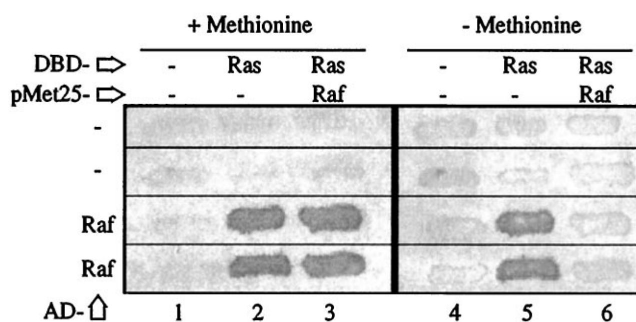

FIG. 3. **β-Galactosidase assay (21) on HF7c yeast cells cotransformed with several couples of plasmids, as indicated.** Cells were selected on a minimal selective medium and then patched on a minimal medium in the presence or the absence of methionine. After 24 h at 30 °C, plates were replicated to test for LacZ expression in the absence or the presence of methionine.

immunoprecipitation from whole double transformed yeast cell extract using antibodies directed toward either of the three subunits or toward the HA epitope of the protein expressed under the control of pMet25. Using antibodies directed toward the HA tag cyclin H, we were able to immunoprecipitate from a yeast total extract not only cyclin H but also cdk7 and MAT1 (Fig. 2*C*, see fractions retained on the beads (*B(−)*), *lanes 2*, *6*, and *12*).

Inhibition of the Transcription Activator Formation—The above assay demonstrates how it is possible to identify a third protein that can favor or stabilize interactions between two partners of a complex. We now illustrate, by using our three-hybrid system, how inhibition of a *bona fide* two-hybrid interaction can be detected. Ras and Raf, two oncogenic proteins involved in the transduction of signals that control cell growth and differentiation, were shown to specifically interact each other in a two-hybrid system (9). In our system, the H-Ras (V12) ORF is fused to the GAL4 DNA-binding domain under the control of the ADH promoter in the pGBT9-3H/B vector, whereas the cRaf-1 ORF is introduced under the control of the Met25 promoter, resulting in plasmid [pGBT-Ras/Met-Raf]. The cRaf-1 ORF is also fused with the GAL4 activation domain behind the ADH promoter of the pGAD-GH vector (Fig. 1), which contains the LEU2 selection gene (27).

Using a paper filter assay, we tested β-galactosidase expression on HF7c yeast cells transformed with a combination of two plasmids (Fig. 3). Cells cotransformed with pGBT9-Ras and pGAD-Raf (data not shown) as well as cells cotransformed with [pGBT-Ras/Met-0] and pGAD-Raf (Fig. 3, *lanes 2* and *5*) show similarly a $LacZ^{+}$ phenotype in the presence or in the absence of methionine. These cells are also prototrophic for histidine (phenotype His^{+}), indicating the expression of the second reporter gene product (data not shown). Hence, *per se* the presence or the absence of methionine in the medium and the presence of the Met25 cassette in a pGBT plasmid do not influence results of two-hybrid tests.

Yeast cells cotransformed with [pGBT-Ras/Met-Raf] and pGAD-Raf are $LacZ^{+}$ in the presence of methionine (condition of repression of the Met25 promoter) but are $LacZ^{-}$ in the absence of methionine (condition of derepression of the Met25 promoter) (Fig. 3, compare *lanes 3* and *6*). The most likely explanation is that c-Raf1, expressed from the Met25 promoter, titrates out enough Ras expressed from [pGBT-Ras/Met-Raf] to preclude the interaction between GAL4DBD-Ras and GAL4AD-Raf, leading to a $LacZ^{-}$ phenotype. Nevertheless, we failed to see a His^{-} phenotype (resulting from the HIS3 gene repression) with [pGBT-Ras/Met-Raf]-pGAD-Raf transformants. This probably reflects the well known higher sensitivity of the histidine reporter gene compared with the β-galactosidase reporter gene in strain HF7c. The latter reporter gene seems much more stringent than the former in terms of effective transactivating complex. It would not be surprising, although it was not tested, if the use of pGAD424-like plasmids instead of the pGAD-GH plasmid expressing Raf (the present study) would allow the use of a growth test to detect the inhibition of the interaction. Actually, pGAD424 drives expression of proteins fused to GAL4 activation domain from a truncated ADH promoter, and it has been shown to give a weaker response in two-hybrid tests (29). Together, these data show that the third polypeptide cRaf-1 prevent the formation of the transcriptional activator.

DISCUSSION

The present study shows the efficiency of a three-hybrid system based on the regulation of a transcriptional activator complex formation. On one hand, the formation of the activator can be promoted by the presence of a third partner that could interact with any of the two other polypeptides, as demonstrated by the reconstitution of the TFIIH-CAK complex. Thus, one can detect complex formation in which the third polypeptide can either bridge or stabilize the two other fusion polypeptide partners. On the other hand, the third protein can also prevent the interaction between the two components of the reconstituted activator by a squelching process, as shown by the Ras-Raf interaction experiment. However, it could not be excluded, for exemple, that a modification (*e.g.* a phosphorylation or a methylation) of any of the two fusion proteins by the third overexpressed polypeptide may simply favor the transcriptional activator complex formation or prevent its formation. In this case, an interaction is detected, although no ternary complex is formed (14), or an inhibition is detected, although no stable interaction between the inhibitor and one of the fused protein is created. Eventually such mechanisms could be of biological significance.

One of the main advantages of our system resides in the presence of the Met25 promoter, regulating the transcription of the cDNA for the third polypeptide, which could be repressed upon the addition of methionine in the culture medium. The presence of a methionine-regulated promoter provides an "on" or "off" switch for the expression of the third protein. As a negative control, by replica plating the colonies on a methionine rich medium, the growth is switched off when the third partner is required for activator formation. In the same way, the growth of colonies on medium lacking methionine is switched off when the third protein has an inhibitory effect on the complex formation. No additional experiment involving a plasmid lacking the third partner cDNA is required.

Our system permits the screening of a cDNA library to identify protein promoting or preventing interaction between two identified polypeptides. The present three-hybrid constructs, all of which contain a DBD and a TRP1 selection gene, are derived from already existing two-hybrid plasmids. To identify the third partner, commercially available libraries that contain the activator element can be easily used, thus avoiding the need of any supplementary library construction. Furthermore, the identification of a third protein by library screening is facilitated by the present three-hybrid system. Actually, our system offers three different ways to discard false positives: (i) detection by amino acid prototrophy, (ii) 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside transformation in a blue component, and (iii) selection of the clones that are unable to grow in the presence of methionine.

In conclusion, we demonstrate here the ability of a three-hybrid system to detect proteins involved in promoting or inhibiting a complex formation, as well as identify interacting domains of protein complexes. Thus, a possible pharmacologi-

cal application of our set of plasmids could be the design of peptide drugs that inhibit an interaction between proteins that are positive in two-hybrid tests (which become the “interacting baits”). Such a system might require the expression of a degenerated oligonucleotide library under control of the Met25 promoter. The inhibition of the interaction would be detected in a growth or in a β-galactosidase test. Under repression (in the presence of methionine) of the Met25 promoter, the $LacZ^+$ or His^+ phenotype should be restored, thus demonstrating that the inhibition is due to expression of the protein under the control of the Met25 promoter and not to other events such as a mutation in one of the interacting baits. Such a system could be elegantly complementary of the reverse two-hybrid system recently published (11) that offers positive selection when interacting proteins stop to interact. Furthermore, a four-hybrid system using a second inducible promoter and a reverse three-hybrid can be easily settled.

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