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# Antibody-promoted dimerization bypasses the regulation of DNA binding by the heme domain of the yeast transcriptional activator HAP1

**(heme regulation/antibody)**

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ABSTRACT **The yeast transcriptional activator HAP1 contains a DNA-binding domain homologous to the zinc finger of GAL4 and an adjacent regulatory domain that blocks DNA binding in the absence of the inducer heme. We show that short HAP1 fragments containing the zinc finger are unable to bind to DNA but can be rescued by antibody to the HAP1 zinc finger. These fragments are missing a coiled-coil sequence similar to that within the dimerization domain of GAL4 and dimerization domains of myosin heavy chain. We surmise that the antibody promotes DNA binding by bringing together two monomers. Interestingly, the antibody will also promote DNA binding of a larger HAP1 fragment containing the DNA-binding and the heme-regulatory domains. This suggests that the regulatory domain acts by preventing dimerization of HAP1 in the absence of heme. Consistent with this view is an *in vivo* assay that also reveals that heme promotes HAP1 dimerization in yeast cells.**

Many transcription factors are DNA-binding proteins that form protein dimers. Dimerization is very important in promoting the functional interaction between members within families of transcription factors (for a review, see ref. 1). In yeast a large set of transcriptional activators, exemplified by GAL4 and HAP1, all have homologous zinc fingers at their amino termini, which dictate DNA binding (2–4). However, the HAP1 activator possesses several properties that distinguish it from GAL4 and other members of this family (5). First, the protein binds to two different classes of sequence elements. Second, the ability of HAP1 to bind to DNA is activated by heme. *In vitro*, HAP1 will not bind to DNA unless heme is included in the reaction. *In vivo*, HAP1 will not activate transcription in heme-deficient mutants grown in the absence of a heme supplement. The heme regulatory domain of HAP1 was mapped between residues 244 and 444 of the protein (3). Deletions that remove the heme domain display constitutive binding to HAP1 DNA sites. Thus, the role of the heme domain is to repress DNA binding in the absence of heme.

In this report, we probe the function of the heme domain of HAP1 by using an antibody-induced DNA-binding approach. We find that short HAP1 fragments presumed to be missing a dimerization domain can be induced to bind to HAP1 DNA-binding sites by HAP1-specific antibody. We are able to locate precisely a putative dimerization domain of HAP1 in this way. Further, we show that HAP1 dimerizes *in vivo* and *in vitro* by using the dihybrid method of Fields and Song (6) in yeast cells and mixing different purified HAP1 size variants, respectively. Finally, we show that the antibody bypasses *in vitro* repression of DNA binding by the heme domain in the absence of heme.

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## MATERIALS AND METHODS

**Yeast Strains and Methods.** *Saccharomyces cerevisiae* strains used were GGY1::171 (7) and LPY22 (8). β-Galactosidase activity was assayed as described (6). Yeast extracts and HAP1 truncation products were prepared as described (3, 9).

**Generation of HAP1 Antibodies.** Two plasmids encoding trpE–HAP1 fusion proteins were constructed. Plasmid p2-3 was constructed by subcloning the *Sma* I–*Hin*dIII (amino acids 1–115) fragment of SD5-HAP1 (10) into the *Sma* I–*Hin*dIII site of pATH2 (11), and plasmid p7-8 was constructed by subcloning the *Bam*HI–*Bgl* II (amino acids 445–749) fragment of SD5-HAP1 into the *Bam*HI site of pATH-3 (11). The fusion proteins were synthesized, separated by a preparative SDS/polyacrylamide gel, and electroeluted with a Bio-Rad electroeluter. The proteins were injected into rabbits according to standard techniques (12). Antibodies were shown to be specific for HAP1 by Western blot analysis. However, only the antibody generated from the fusion protein by plasmid p2-3 was found to promote the supershifting of HAP1–DNA complexes and used in the experiments described here.

**Construction of GAL4 DNA-Binding Domain and Activation Domain Plasmids.** To construct GAL4 activation domain–HAP1 fusion plasmids, SD5-HAP1 (10) was digested with *Bam*HI, and the 1344-bp HAP1 fragment was inserted into plasmid GAD10 (13) digested with *Bam*HI. To construct GAL4 DNA-binding domain–HAP1 plasmids, SD5-HAP1 was digested with *Sma* I and *Bam*HI, and the 1336-bp HAP1 fragment was ligated with a *Bgl* II linker (GAAGATCTTC). Then, the fragment was digested with *Bgl* II and inserted into plasmid MA 424 (14) digested with *Bam*HI.

**DNA-Binding Assays.** The radiolabeled UAS1/CYC1 probe was prepared as described (8). The binding reaction mixtures contained 5% glycerol, 4 mM Tris (pH 8), 40 mM NaCl, 4 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM $Zn(OAc)_2$, 3 μg of salmon sperm DNA, ≈1 ng of labeled probe, and 15 μg of protein extracts or 4 μl of *in vitro*-synthesized HAP1 fragment. Heme was added to a final concentration of 20 ng/μl. The appropriate amount of HAP1 antibody or preimmune serum was added to the reaction after a 25-min incubation at room temperature. Then, the reaction mixtures were incubated for another 25 min and loaded immediately onto a 4% or 6% polyacrylamide gel in TBE buffer as described (3).

## RESULTS

**Antibody-Promoted DNA Binding.** Antibodies were tested for their ability to supershift HAP1–DNA complexes as observed by gel shift assay. In Fig. 1, we show that heme promotes HAP1 binding to a UAS1 probe, yielding complex B (compare lanes 3 and 6). In a control strain not bearing the HAP1 plasmid, no HAP1 complexes were seen (compare lanes 1 and 2). While preimmune serum had no effect on the HAP1–DNA complex (lane 5), the HAP1 antibody to the zinc finger gave rise to two new complexes of altered mobility, A

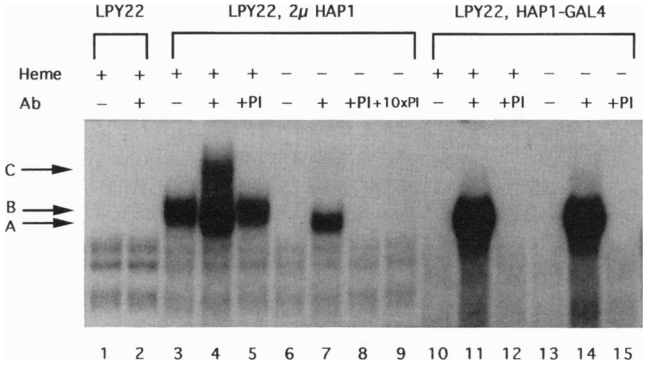


FIG. 1. Effect of the HAP1 antibody on HAP1 binding at the UAS1/CYC1 element. HAP1 antibody (Ab) (lanes 2, 4, 7, 11, and 14) or preimmune (PI) serum (lanes 5, 8, 9, 12, and 15) was added to extracts prepared from HAP1-deleted LPY22 cells (lanes 1 and 2), LPY22 cells bearing the 2-μm HAP1 plasmid (lanes 3–9), and LPY22 cells bearing a plasmid encoding HAP1 (residues 1–247)–GAL4 (residues 753–881) fusion protein (lanes 10–15). The reaction mixture also contained the UAS1 probe in the presence (lanes 1–5 and 10–12) or absence (lanes 6–9 and 13–15) of heme. In lane 9, 10 times more concentrated preimmune serum was added. The amount of preimmune serum was normalized to the amount of antibody according to the amount of total protein. The same pattern of complexes was observed in a parallel experiment in which the CYC7 probe was used (not shown).

and C (lane 4). Antibody to the internal HAP1 domain did not supershift the HAP1–DNA complex (data not shown). Complex C was presumed to be the supershifted HAP1–DNA complex, but the identity of complex A was not immediately apparent.

We inferred the identity of complex A from several observations. First, its appearance required both the HAP1 expression plasmid and the specific anti-HAP1 serum. Second, its mobility was much faster than complex C, suggesting that it could represent a fragment of HAP1 in a complex with the antibody. Surprisingly, we found that the antibody, but not preimmune serum, promoted the formation of complex A in the absence of heme (lanes 6–9). Complex C was not observed in the absence of heme. This finding suggested that complex A contained a fragment of HAP1 that did not extend to include the heme domain. This idea was further tested by the examination of a HAP1–GAL4 fusion protein (15). This fusion contained the DNA-binding domain of HAP1 (residues 1–244) fused to residues 753–881 of GAL4 but was missing the heme domain. When synthesized *in vitro* this fusion bound to UAS1 constitutively (10). In yeast extracts, we did not observe binding of HAP1–GAL4 in the absence or presence of heme (lanes 10, 12, 13, and 15), suggesting that the fusion protein was not stable in yeast extracts. However, the HAP1 antibody promoted the formation of high levels of complex A in the HAP1–GAL4 extract (Fig. 1, lanes 11 and 14). We have also observed the promotion of formation of complex A in extracts containing other unstable HAP1 fusion proteins, although its amount varies with different fusion proteins (data not shown). These findings strengthen the surmise that complex A contains a short fragment of HAP1 and the HAP1-specific antibody.

**HAP1 Dimerizes *in Vivo* and *in Vitro*.** We considered two models for how antibody promotes the binding of HAP1 fragments to UAS1. In the first, we imagined that the HAP1 fragment was missing the HAP1 dimerization domain. The antibody promotes DNA binding by bringing two HAP1 monomers into close proximity to one another by this model. In the second, the antibody induces a conformational change in the fragment to stabilize its binding to DNA. A minimum condition by model one is that HAP1 bind to DNA as a dimer. We therefore turned to the *in vivo* approach of Song and Fields (6, 13) to test whether HAP1 could dimerize. In this method two hybrid proteins were synthesized *in vivo*. One contained the DNA-binding domain of GAL4 fused to HAP1 residues 1–444. The second contained the acidic transactivation domain of GAL4 fused to HAP1 residues 1–444. Plasmids were transformed into a $Hem^+$ strain, which synthesizes heme endogenously and thus activates HAP1. If the HAP1 domains of the two hybrids dimerized, then the acidic domain of GAL4 would be brought to the DNA-binding domain of GAL4, reconstituting the functional transcriptional activator. Indeed, cells bearing both hybrids yield significant activation of a GAL4-responsive reporter (Fig. 2*A*). These findings demonstrate that residues 1–444 of HAP1 promote dimerization *in vivo*. Below, we use this system to demonstrate that dimerization is regulated *in vivo*.

To further demonstrate that HAP1 dimerizes *in vitro* as well, two size variants of HAP1 encompassing amino acids 1–171 and 52–171 were synthesized in *E. coli* and purified (details will be described elsewhere). As shown in Fig. 2*B*, both peptides are able to bind to DNA (lanes 1 and 6). When the two peptides were mixed prior to DNA-binding reactions, a third band with intermediate mobility of the original two HAP1 bands appears (Fig. 2*B*, lanes 2–5). The only explanation for this is that the new band represents the heterodimer of the two peptides while the original two bands are homodimers of the two HAP1 derivatives. Thus, this result demonstrates that HAP1 also dimerizes *in vitro*.

**Mapping the Dimerization Domain of HAP1.** If the HAP1 antibody promoted DNA binding by causing dimerization, we might use the serum as a reagent to map the HAP1 dimerization domain. From previous results (3), the dimerization domain of HAP1 must lie within the first 148 residues of the protein. HAP1 fragments were synthesized *in vitro* and examined for binding to a UAS1 probe in the absence or presence of HAP1 antibody. Fragments containing residues 1–247, 1–174, or 1–148 bound in the absence or presence of antibody (Fig. 3). However, fragments 1–123 and 1–115 bound only in the presence of the antibody. A shorter fragment of HAP1 containing residues 1–100 did not bind to DNA even in the presence of antibody (data not shown). These findings indicate that the 1–115 and 1–123 HAP1 fragments contain sequences for specific binding to UAS1 but are missing a dimerization domain. Fragments 1–148, 1–174, and 1–247 contain the dimerization domain. Essential sequences of the HAP1 dimerization domain are therefore contained between residues 123 and 148 of HAP1.

**A Conserved Dimerization Motif.** It was suggested that the GAL4 dimerization domain bore similarity to a region of HAP1 that was spaced 7 residues further from the zinc finger homologous region (4). Fig. 4 shows this alignment between HAP1 residues 100–135 and GAL4, as well as other members of this family of transcriptional activators, LAC9, Qa1F, and MAL63 (2, 16–18). Although the degree of conservation is not nearly as striking as the conservation in the zinc finger regions, 6 hydrophobic residues are highly conserved in HAP1 and GAL4. These 6 residues form the 4-3 hydrophobic repeat characteristic of coiled coils, providing a major thermodynamic driving force for dimerization (19). The conservation of these hydrophobic residues in HAP1 and GAL4 suggests that this region of HAP1 also forms a coiled coil like that of GAL4. Furthermore, a search of the GenBank, European Molecular Biology Laboratory, Protein Identification Resource, and Transcription Factor data bases identified several proteins with a more extensive degree of similarity to the dimerization domain of HAP1. Strikingly, these other sequences are all from the tails of the myosin heavy chains from *Drosophila* (20), chicken (21), and *C. elegans* (22). The rod-like tails of myosin heavy chains contain repeats of 28 residues with alternating hydrophobic and charged residues (23, 24). These repeats allow the tails of myosin heavy chain to form two-stranded α-helical coiled coils, thus promoting

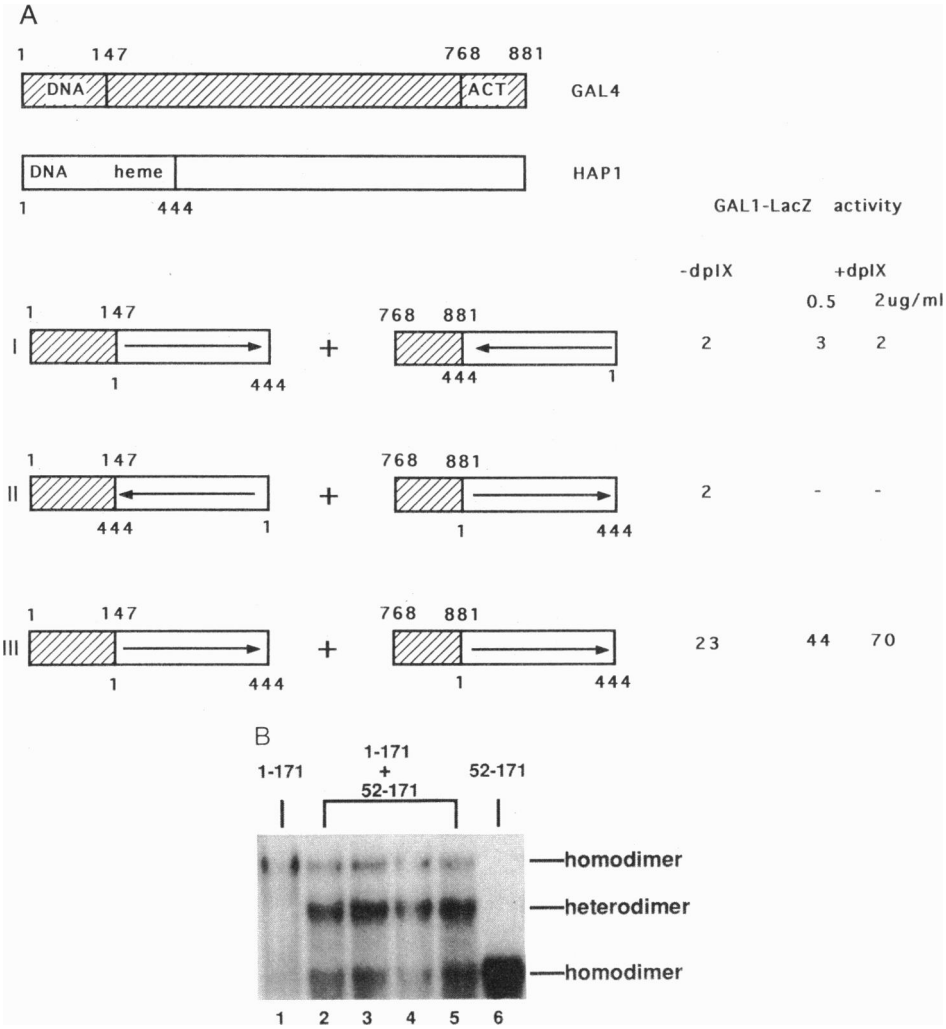


FIG. 2. (*A*) Reconstitution of GAL4 activity by HAP1–HAP1 hybrids. The HAP1-(1–444) fragment encompassing the DNA-binding (DNA) and heme-responsive (heme) domains was fused to the GAL4 DNA binding domain (DNA) and the GAL4 activation domain (ACT) separately. Row III shows the GAL1–lacZ activity resulting from the coexpression of both fusion proteins. As a control, GAL1–lacZ activity was also assayed when the orientation of HAP1 coding sequences was reversed in the GAL4 activation domain fusion (row I) or the GAL4 DNA binding domain fusion (row II). The effect of a heme analog, deuterophorphyrin IX (dpIX), on the reconstituted GAL4 activity is shown in row III. Note that both fusions are under control of the constitutive ADH1 promoter. (*B*) HAP1 dimerizes *in vitro*. Two size variants of the HAP1 DNA-binding domain encompassing amino acids 1–171 (lane 1) and 52–171 (lane 6) were generated in *Escherichia coli*. The two purified peptides were mixed together and incubated at 25°C (lane 2), 37°C (lane 3), 42°C (lane 4), and 55°C (lane 5) for 30 min prior to DNA-binding reactions.

dimerization and polymerization (23, 24). The highly conserved residues include the hydrophobic residues in 4-3 repeats and charged residues. These charged residues could form both intra- and interhelical ion pairing and provide significant energy for forming coiled coils and dimerization (19). The conservation of these residues underlines their functional importance. All these data suggest that HAP1 and myosin heavy chains use similar sequences for protein–protein interaction and dimerization.

**Role of Heme in HAP1 Dimerization.** As described above, heme promotes DNA binding of HAP1 by counteracting an internal repression sequence between residues 244 and 444 of HAP1 (3). We wished to investigate whether this regulation of DNA binding by heme occurred at the level of HAP1 dimerization. If the heme domain prevented dimerization in the absence of heme, then it might be possible to bypass the heme requirement for binding by addition of the HAP1 antibody. We expressed a HAP1 derivative (residues 1–444) containing the DNA binding and heme domain in yeast cells and examined the DNA-binding properties of this derivative in yeast extracts. Fig. 5 shows that the DNA binding of the HAP1 fragment was strongly induced by heme in the absence of antibody to form complex D (compare lanes 1 and 3). Addition of the antibody in the presence of heme gave rise to two new complexes. Complex E migrated in the same position as complex A in Fig. 1 and is presumed to be the short fragment of HAP1 (complex A in Fig. 1). Complex F migrates more slowly and is presumed to contain the HAP1-(1–444) fragment, antibody, and DNA.

Strikingly, when antibody was added in the absence of heme, the formation of complex E and a complex with the approximate mobility of F (F′) was observed. The mobility of this latter complex suggested that it corresponded to the HAP1-(1–444) fragment bound to antibody and DNA. These findings indicate that the HAP1 antibody can stimulate the binding of the HAP1-(1–444) fragment of HAP1 to UAS1 in the absence of heme. The slight difference in mobility of F and F′ may be due to an alteration in the conformation of the HAP1-(1–444) fragment upon binding heme. These findings suggest that dimerization of HAP1 is prevented by the heme domain in the absence of heme and that the role of heme is to counteract this block.

We next tested whether heme regulates dimerization of HAP1 *in vivo*. Previous findings indicated that addition of the

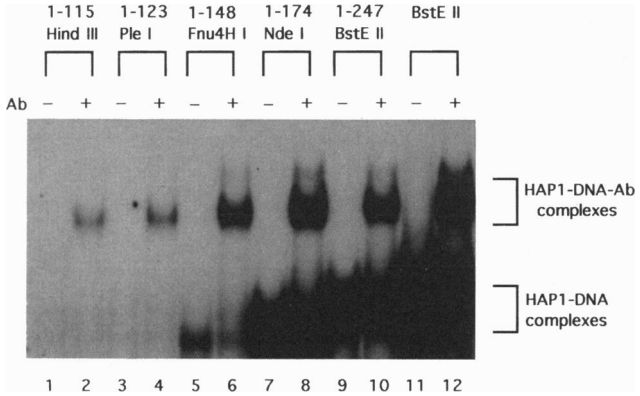


FIG. 3. Effect of HAP1 antibody (Ab) on the binding of *in vitro*-synthesized HAP1 truncation products. Different lengths of HAP1 fragments were produced by digesting the SP6 promoter-driven HAP1 plasmid with restriction enzymes *Hin*dIII, *Ple* I, *Fnu*4HI, *Nde* I, and *Bst*EII, followed by *in vitro* transcription and translation. As a comparison, lanes 11 and 12 show the effect of antibody on the binding of the HAP1 *Bst*EII fragment in yeast extracts prepared from cells bearing the correct plasmid.

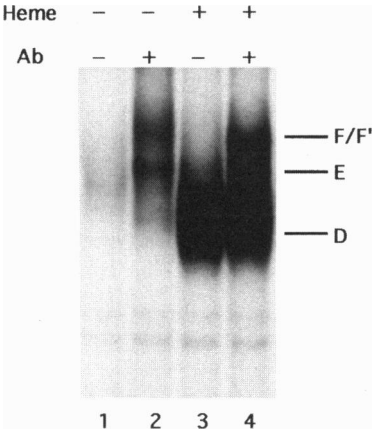


FIG. 5. Effect of antibody (Ab) on the binding of the HAP1-(1–444) fragment bearing the heme-responsive domain. Extracts were prepared from LPY22 cells bearing the HAP1 *Bam*HI frameshift plasmid, which produces the HAP1-(1–444) truncation product. Lanes 1 and 2 show the effect of the antibody to amino acids 1–115 of HAP1 on the binding in the absence of heme; lanes 3 and 4 are identical except for the addition of heme.

heme analog deuterophorphyrin IX stimulated a HAP1 reporter by about 3- to 4-fold (3) above the levels activated by the endogenous synthesis of heme. If this effect occurred at the level of HAP1 dimerization, then the heme analog should induce the activity of the GAL1 reporter driven by the dihybrid constructs. Indeed, deuterophorphyrin IX gave rise to a 3-fold induction of the GAL1 reporter (Fig. 2*A*), consistent with the model that heme stimulates dimerization of HAP1 and that the heme regulatory domain prevents dimerization in the absence of heme.

## DISCUSSION

In this report, we have shown that specific antibodies to the HAP1 activator protein can induce the DNA binding of certain short amino-terminal fragments of the protein. One form of truncated HAP1 was found in extracts from cells that bore the wild-type HAP1 or various HAP1 fusion proteins. Other forms of HAP1 whose DNA binding was rescued by the antibodies were produced by *in vitro* synthesis of amino-terminal fragments of the protein.

We believe that the antibody stimulates DNA binding by bringing together two HAP1 monomers. These monomers consist of protein fragments that retain sequences for site-specific DNA binding but are missing a dimerization domain. Several findings validate this model. First, the results from the *in vitro* experiment mixing size variants of HAP1 and the *in vivo* system of Fields and Song (6) unequivocally demonstrated that HAP1 dimerizes when functional. Second, the amino-terminal fragments whose DNA binding was stimulated by the antibody contain the GAL4 homologous zinc finger of HAP1 but are missing a region similar to the coiled coil of GAL4, which promotes its dimerization. In addition, because many lines of evidence suggest that sequences within zinc finger domains are sufficient for determining protein folding (4, 25), we do not favor the model in which the antibody stabilizes a HAP1 conformation suitable for DNA binding.

Using the antibodies, we were able to locate residues important for dimerization between amino acids 123 and 148 of HAP1. This region also bore similarity to other yeast activators of the GAL4 family, including LAC9, Qa1f, and MAL63 (2, 16–18), and heavy chains of myosin from several species, which form coiled-coil structures and facilitate protein–protein interaction (20–24).

In the intact HAP1, the dimerization region is followed by sequences that mediate induction by heme. This region (between residues 244 and 444) represses DNA binding *in vitro* unless heme is added to the reaction (3). How does the heme domain of HAP1 repress DNA binding in the absence

| | | |
|---|---|---|
| **HAP1** | 100 | **W A E E A E K E L L K D N E D K K D R E R V K S D E K T D S K V H S S P** |
| **GAL4** | 40 | **Y - - - - - R - - L = = = = L = = V = = R L = = L E - - F - - I** |
| **LAC9** | 156 | **F - - - - - - - L L - Q - D - - R I R E - L - G - - - T V - - L - S** |
| **Qa1f** | 112 | **R G - Q - G - - - - - E - - L - - M - E - V - - - E - - L - - L** |
| **MAL63** | 41 | **- - - - - - - - - - I R - - - L K K I - E - M - S M N - - I** |
| **Myo.** | 891 | **- - - Q - E K - V - K E D E L K - V R E K L - T L - K** |
| **Dro.** | 1761 | **- A N N A N K G - L - - D E - R R L - - R I - - L E - - L** |
| **Myo.** | 463 | **- - E E - E R N - I - Q N E - K K M Q - H I - - L E - - L** |
| **Ckn.** | 379 | **- - - Q - E - - - - K D E E L - K V K E K - - - V E - - L - - M** |
| **Myo.** | 859 | **E E L - K I - D K V K A L E - S L A K** |
| **Cel.** | 956 | **E - E V - - L K - - I - - L E - - L - K - - S** |

FIG. 4. Alignment of HAP1 sequences 100–135 with the GAL4 dimerization sequences and *Kluyveromyces lactis* regulatory protein LAC9 (14), *Saccharomyces cerevisiae* maltose activator protein MAL63 (15), *Neurospora crassa* regulatory protein Qa1f (16), as well as the rod-like tails of myosin heavy chains from *Drosophila melanogaster* (Myo. Dro.; ref. 17), chicken (Myo. Ckn.; ref. 18), and *Caenorhabditis elegans* (Myo. Cel.; ref. 19). Only conserved amino acids are shown. Identical amino acids are shown in boldface type. No gaps are present in these alignments. The shadowed region in GAL4 (residues 50–64) forms α-helical coiled-coil structure and serves as part of a dimerization element of the intact protein. Six highly conserved hydrophobic residues in HAP1 are circled, and seven highly conserved charged residues among HAP1 and myosin repeats are underlined in HAP1 sequences.

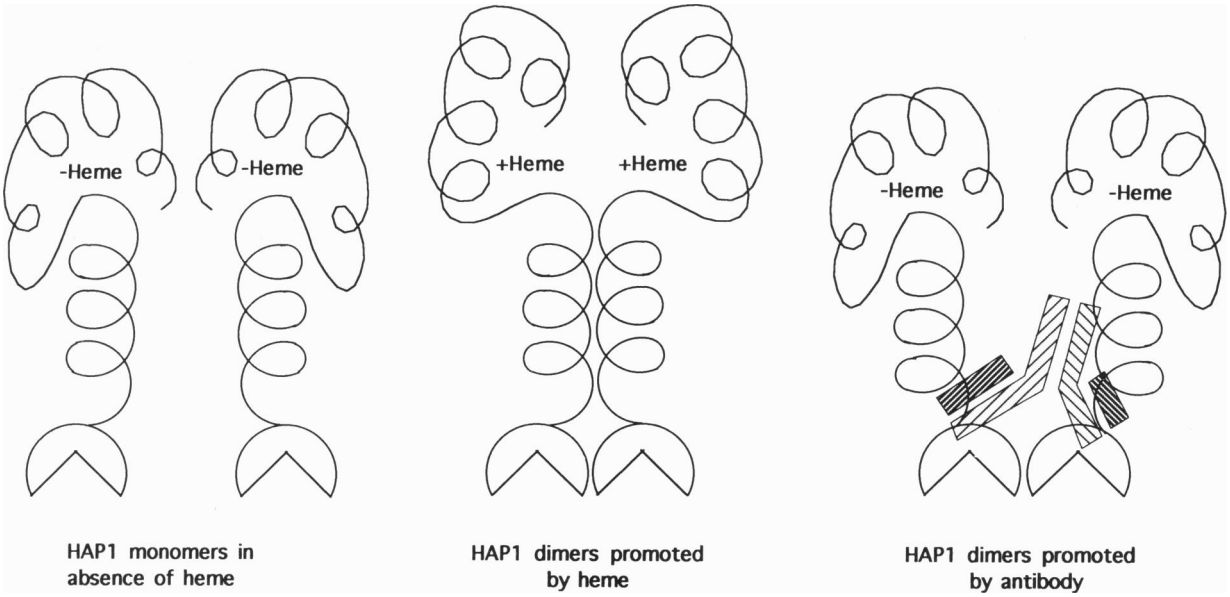


FIG. 6. Model for how heme and antibody may promote dimerization. The zinc finger DNA-binding domain is shown as a wedged circle. The coil represents the putative dimerization domain, and the heme-responsive domain is shown in random coils. Hatched boxes represent the heavy and light chains of an IgG dimer. In the absence of heme, the heme domain blocks the dimerization domain. This block may be alleviated by addition of heme, which unmasks the dimerization domain, or HAP1 antibody, which itself promotes dimerization.

of the inducer? We report here the important finding that the HAP1 antibodies bypassed the heme requirement of a HAP1 fragment bearing both the DNA-binding and heme domains. This finding suggests that the reason that HAP1 does not bind in the absence of heme is that it does not dimerize. Thus, the heme domain of the protein blocks dimerization in the absence of heme. Using the dihybrid system of Fields and Song (6), we found that heme also regulated HAP1 dimerization *in vivo*.

In Fig. 6 we indicate repression of DNA binding in the absence of heme as occurring by an intramolecular interaction involving the heme and DNA-binding domains of HAP1. Alternatively, it is possible that the heme domain binds to another protein and that this second protein prevents oligomerization. This regulation of dimerization by a small signaling molecule is similar to the regulation of the membrane-bound epidermal growth factor (EGF) receptor (26) and the nuclear estrogen receptor (27). Binding of EGF to cell surface EGF receptors initiates the formation of the receptor homodimers and internalization, whereas binding of heme to the heme domain promotes HAP1 dimerization and DNA binding.

In summary, the regulation of assembly of oligomeric proteins is a powerful way to exert tight control on protein activity. The use of specific antibodies to bypass regulatory blocks of dimerization may be a general way to identify other cases of this form of regulation. It is conceivable that in certain cases the antibody-promoted bypass will facilitate a more efficient process than is ever observed under normal conditions.

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