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The C6 zinc cluster dictates asymmetric binding by HAP1

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Unlike other C6 zinc cluster proteins such as GAL4 and PPR1, HAP1 binds selectively to asymmetric DNA sites containing a direct repeat of two CGG triplets. Here, we show that the HAP1 zinc cluster is solely responsible for asymmetric binding by HAP1. An asymmetric interaction between two zinc clusters of a HAP1 dimer must position the zinc clusters in a directly repeated orientation, and enable them to recognize two CGG triplets in a direct repeat. Further, our data suggest that this asymmetric interaction acts cooperatively with the interaction between dimerization elements to promote HAP1 dimerization, and locks HAP1–DNA complexes in a stable, dimeric conformation.

Keywords: activator/asymmetric binding/dimerization/HAP1/zinc cluster

Introduction

The yeast C6 zinc cluster proteins are a family of transcriptional activators that control a wide variety of processes ranging from nucleotide synthesis to oxygen utilization (Johnston, 1987). For example, GAL4 controls transcription of genes required for metabolism of galactose and melibiose (Oshima, 1982); PPR1 controls pyrimidine biosynthesis (Loison *et al.*, 1980; Roy *et al.* 1990); and HAP1 controls transcription of genes involved in respiration such as cytochromes (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989). There are 39 of these C6 zinc cluster proteins that have been identified to date (Gardner *et al.*, 1995). Those characterized biochemically, including GAL4, HAP1, PPR1 and PUT3, all bind to DNA as dimers (Carey *et al.*, 1989; Siddiqui and Brandriss, 1989; Roy *et al.*, 1990; Zhang *et al.*, 1993). Their DNA binding domains contain three elements (Marmorstein *et al.*, 1992; Reece and Ptashne, 1993; Marmorstein and Harrison, 1994): the C6 zinc cluster motif, a coiled-coil dimerization element and a linker that connects the dimerization element to the zinc cluster (Figure 1A). The C6 zinc clusters are very well conserved in the family (Johnston, 1987), and Lys41 and Lys43 that make specific base contacts in GAL4 or PPR1 (Figure 1A) correspond to Lys71 and Lys73 in HAP1 (Johnston, 1987; Creusot *et al.*, 1988; Pfeifer *et al.*, 1989; Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). The only major difference between the zinc clusters of HAP1 and PPR1 is the altered spacing between the fifth and the sixth Cys residues: HAP1 contains two extra residues, HL, in this region (Figure 1A).

There are two interesting ways in which different DNA binding specificity occurs among these proteins. The first is specificity due to spacing between the CGG triplets. Data from X-ray crystallography show that the six Cys residues in GAL4 and PPR1 bind to two zinc ions and form a compact, rigid, binuclear cluster (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). The C6 binuclear cluster recognizes a CGG triplet in the cognate DNA sites. GAL4, PPR1 and PUT3 all recognize DNA sites containing two rotationally symmetric CGG triplets, but the two triplets of these sites are separated by different numbers of base pairs (Siddiqui and Brandriss, 1989; Roy *et al.*, 1990; Reece and Ptashne, 1993). The linker and the beginning of the dimerization element of these proteins are responsible for directing the proteins to their sites with preferred spacing (Reece and Ptashne, 1993).

The second determinant of specificity is orientation of the CGGs. Strikingly, HAP1 recognizes an asymmetric, direct repeat of two CGG triplets separated by six nucleotides: CGGnnnTnnCGG (Zhang and Guarente, 1994). Further, footprinting data show that the two zinc clusters of a HAP1 dimer must be positioned asymmetrically in a directly repeated orientation to make the same contacts with the two CGG triplets in a direct repeat (Zhang and Guarente, 1994). How can the zinc clusters of a HAP1 homodimer be positioned asymmetrically to recognize an asymmetric DNA site? Previously, we have shown that the HAP1 dimerization element is not responsible for HAP1 asymmetric binding because it can be substituted by the PPR1 dimerization element without affecting the ability of HAP1 to bind preferentially to asymmetric DNA sites (Zhang and Guarente, 1994). Inferring from previous results on GAL4, PPR1 and PUT3 (Reece and Ptashne, 1993), we proposed that the linker of HAP1 is very likely responsible for asymmetric binding (Zhang and Guarente, 1994).

Surprisingly, we show here that the C6 zinc cluster, not the linker, is solely responsible for asymmetric binding by HAP1. We made various hybrid fragments containing PPR1 and HAP1 sequences, and we found that the HAP1 zinc cluster provides an asymmetric interaction that not only permits the two subunits of a HAP1 homodimer to be positioned asymmetrically, but is also required for stable dimerization. We present a model illustrating the molecular events leading to HAP1 dimerization and asymmetric DNA binding.

Results

To determine which element of the HAP1 DNA binding domain is responsible for HAP1 binding to asymmetric DNA sites, we made two classes of hybrid fragments containing PPR1 and HAP1 sequences. We chose PPR1 because the PPR1 site also contains a six nucleotide spacer

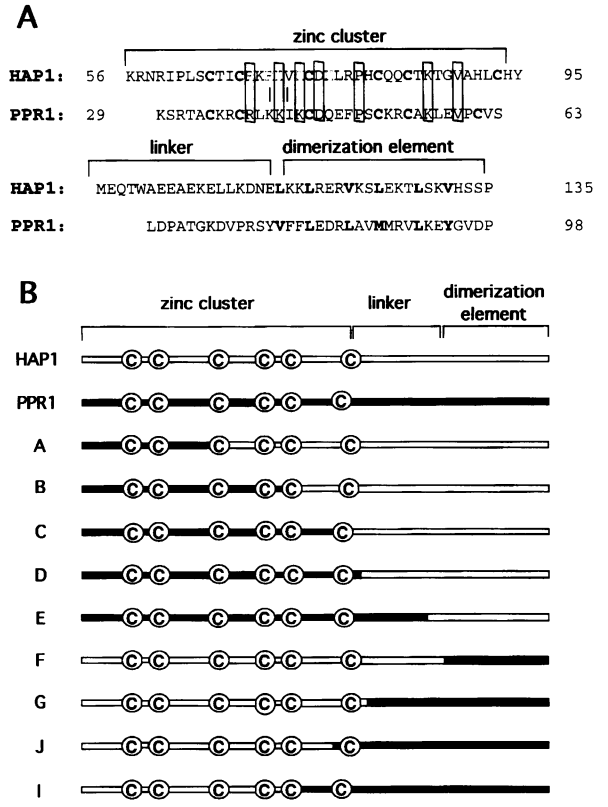

Fig. 1. (**A**) Partial primary amino acid sequences of HAP1 and PPR1. Shown are the zinc clusters, the linkers and the coiled-coil dimerization elements of HAP1 and PPR1. The Cys residues in the zinc clusters and hydrophobic residues in the 4–3 hydrophobic repeat (Cohen and Parry, 1990) of the dimerization elements are all shown in bold face. The conserved residues in HAP1 that are shown to contact CGG in PPR1 or GAL4 are outlined (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989; Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). The identical residues in the zinc cluster elements of HAP1 and PPR1 are boxed, while the conserved residues are shown by lines. (**B**) Amino acid sequences of HAP1–PPR1 hybrid proteins. Each hybrid protein is shown diagrammatically with HAP1 sequences in white and PPR1 sequences in black.

(Reece and Ptashne, 1993; Zhang and Guarente, 1994). The first class of fragments described below contain HAP1 residues in their N-terminus and PPR1 residues in their C-terminus (F–I, Figure 1B). The second class of fragments contain PPR1 residues in their N-terminus and HAP1 residues in their C-terminus (A–E, Figure 1B). These fusions reveal several unexpected properties of HAP1.

The C6 zinc cluster is necessary and sufficient for HAP1 binding to asymmetric DNA sites

Among the first class of fragments described below, both fragments F (containing the HAP1 zinc cluster and linker but the PPR1 dimerization element, Figure 1B and Table I) and G (containing the HAP1 zinc cluster, but the PPR1 linker and dimerization element, Figure 1B and Table I) bind selectively to the HAP1 site but not the PPR1 site (Figure 2, lanes 7, 8, 17 and 18), although the affinity of G is ~2- to 3-fold lower than F. Next, we studied the specificity of DNA binding in greater detail by using the following mutant sites: M1, which mutates one of the CGGs to CCG; M2, which mutates the central conserved T to C; and M3, which has both changes. These mutations all weaken binding of HAP1, as well as the G and F fusions (Figure 3, lanes 1–15). The greater binding of F to the M2 site probably results from the presence of the stronger PPR1 dimerization domain, a point reinforced in experiments below. Therefore, these results, contrary to previous expectation (Zhang and Guarente, 1994), demonstrate that the zinc cluster, not the linker, must provide asymmetric interactions required for positioning the two zinc clusters in a directly repeated orientation.

Which residues of the HAP1 zinc cluster are involved in the asymmetric interaction? As mentioned above, the major difference between HAP1 and PPR1 zinc clusters is the altered spacing between the fifth and the sixth Cys residue of HAP1 (Creusot *et al.*, 1988; Pfeifer *et al.*, 1989; Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). To test whether the altered spacing is critical for the asymmetric interactions, we made two mutations in this region in fusion G: His91 to Cys and Cys93 to Ala to create the same spacing between the fifth and sixth Cys residues as in PPR1. The resulting protein H (Table I) completely lost its ability to bind to DNA. Similarly, we constructed two more fusions in which the residues around the sixth Cys were replaced by the corresponding residues of PPR1 (I = HAP1 residues 56–81 + PPR1 residues 52–123; J = HAP1 residues 56–90 + PPR1 residues 61–123, Figure 1 and Table I). Like the mutant H described above, these two fusions do not bind to either the HAP1 site or the PPR1 site (Figure 2, lanes 10, 11, 20 and 21). Together, these results suggest that the altered spacing of Cys residues in HAP1 may be critical for the asymmetric interactions and DNA binding of the proteins.

The HAP1 zinc cluster also helps promote dimerization when HAP1 binds to DNA

Among the second class of hybrid fragments, containing PPR1 residues in the N-terminus and HAP1 residues in the C-terminus (Figure 1 and Table I), only A and E bind to DNA specifically, while B, C and D only associate with DNA non-specifically in the absence of competitor DNA (not shown, can also be observed for D in lanes 5 and 15, Figure 2). Interestingly, A and E bind to both the HAP1 site and the PPR1 site with the same affinity (Figure 2, lanes 2, 6, 12 and 16; also Figure 3, lanes 16 and 20). Further, the mobility of A–DNA and E–DNA complexes is faster than the dimeric HAP1–DNA complex and near a minor band generated presumably by monomer HAP1 binding (comparing lanes 2 and 6 with 1, Figure 2). These results suggest that A and E bind to DNA as a monomer.

To obtain additional evidence that the faster migrating band corresponds to monomer binding, we carried out a titration of HAP1 binding to wild-type or mutant sites. In Figure 4A, we observe that the HAP1–DNA complex migrates as a dimer at all concentrations tested (lanes 10–12). However, when we use the PPR1 site (PP), a spacer mutant inserting an additional three nucleotides between the CGGs (N3) or a mutant site with only one CGG (TA), the predominant species observed migrates at the faster position, further indicating that it is a monomer. Only at the highest concentrations of HAP1 do we observe complexes migrating at the dimer position, corresponding to the binding of two monomers. We draw three conclusions from this experiment. First, the faster migrating species corresponds to monomer binding. Second, HAP1 must exist predominantly as a monomer in solution. Third,

Table I. Summary of the DNA binding properties of HAP1–PPR1 fusions

Fusion	HAP1 sequence	PPR1 sequence	Binding at HAP1 site	Binding at PPR1 site
HAP1	56–148	none	+ (<50 nM)	–
PPR1	none	29–123	–	+ (<50 nM)
A	75–148	29–44	+ (200 nM)	+ (200 nM)
B	85–148	29–54	non	non
C	93–148	29–61	non	non
D	96–148	29–63	non	non
E	118–148	29–80	+ (200 nM)	+ (200 nM)
F	56–117	81–123	+ (<50 nM)	–
G	56–95	64–123	+ (100 nM)	–
H	56–95 H91 to C, C93 to A	64–123	–	–
I	56–81	52–123	–	–
J	56–90	61–123	–	–

+ indicates strong binding, and – indicates no binding at all. The concentrations in parentheses are approximate protein concentrations required to bind half of the labeled DNA; this concentration defines the dissociation constant (K_d) (Fairall *et al.*, 1992). HAP1, PPR1 and F required <50 nM to bind to half of the labeled DNA (see also Zhang and Guarente, 1994). Protein concentrations (expressed as molarities of monomer) were estimated by the method of Bradford (1976) with bovine serum albumin as a standard. Non-specific binding is indicated by non. For fusions D and C, the non-specific binding was clear even in the presence of competitor DNA (Figure 2). Fusions B, C and D all showed strong binding to labeled DNA in the absence of competitor (not shown). On the contrary, fusions H, I and J did not show any binding even in the absence of competitor (not shown).

a cooperative interaction on the DNA stabilizes dimer binding. Inserting three nucleotides into the HAP1 site disrupts this cooperative interaction.

In summary, the experiments lead to the important conclusion that the A and E fusions bind to DNA as monomers. A more careful study of the E fusion was carried out by titrating the binding of the protein to wild-type and mutant sites. As shown in Figure 4B, the E fusion bound comparably to HAP1, PPR1 or the spacer mutant sites. In all cases, monomer binding was observed at low protein concentrations, and some dimer binding was observed at the highest concentration. This is what was observed for binding of HAP1 to mutant sites above. We conclude that the E fusion binds as a monomer due to the weak HAP1 dimerization domain and the absence of a cooperative interaction in the PPR1 zinc cluster. The A fusion, by inference, is also missing the cooperative interaction in the zinc cluster required for strong dimer binding.

Finally, monomeric binding by the E fusion provided an insight into the nature of the HAP1 half-site. Mutation in the HAP1 site that changes the direct repeat of two CGGs into an inverted repeat (CGGnnnTnnCGG→ CGGnnnTnnCCG) has no effect on the binding by E (Figure 3, comparing lane 19 with 20). However, when T in the spacer is mutated to C, the affinity is reduced ~2- to 3-fold (Figure 3, comparing lanes 17 and 18 with lanes 19 and 20), suggesting that the T is contacted by the monomer. This T is also present in the PPR1 binding site (Marmorstein *et al.*, 1992; Reece and Ptashne, 1993; Marmorstein and Harrison, 1994), and it also helps PPR1 binding (data not shown). Thus, our data together suggest that the A or E monomer recognizes the half-site CGGnnnT, and not TnnCGG. The fact that a natural HAP1 site, the UAS of *CYC7* (Pfeifer *et al.*, 1987), contains two copies of CGCtatT also supports the idea that the half-site is CGGnnnT.

The above data provide two indications that the HAP1 dimerization domain, although required for high affinity bindings of HAP1 (Zhang *et al.*, 1993), is weak. First, HAP1 binds to sites containing one CGG as a monomer.

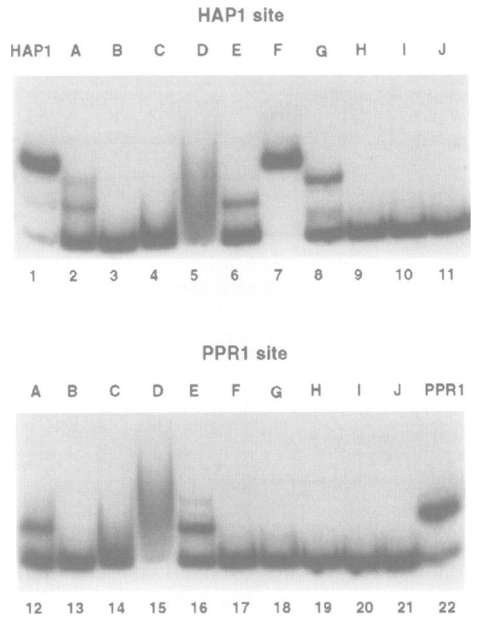

Fig. 2. DNA binding of the HAP1–PPR1 fusion proteins. 50 nM of HAP1, PPR1 or fusion F; 100 nM of G; or 200 nM of A, B, C, D, E, H, I or J were incubated with the HAP1 binding site (CGGACTT-ATCGG) or the PPR1 binding site (CGGCAATTGCCG) in a 20 µl reaction mixture containing 5% glycerol, 4 mM Tris, pH 8, 40 mM NaCl, 4 mM $MgCl_2$, 10 mM dithiothreitol, 3 µg of salmon sperm DNA, 10 µM $ZnOAc_2$, 300 µg/ml bovine serum albumin. Approximately 0.01 pmol of labeled DNA was used in each reaction. The reaction mixtures were incubated at room temperature for 1 h, and then loaded onto 4% polyacrylamide gels in 1/2TBE for gel electrophoresis at 4°C. Under these conditions, PPR1 does not bind to the HAP1 site at all (see Zhang and Guarente, 1994; and data not shown), while HAP1 binds to the PPR1 site as a monomer (see Figure 4A below).

Second, fusions A and E containing the HAP1 dimerization element bind to any sites as monomers. To obtain further evidence that HAP1 is monomeric in solution, we carried out two additional experiments. First, we titrated binding of the F fusion, in which the HAP1 dimerization element has been replaced with that of PPR1, to HAP1 and mutated

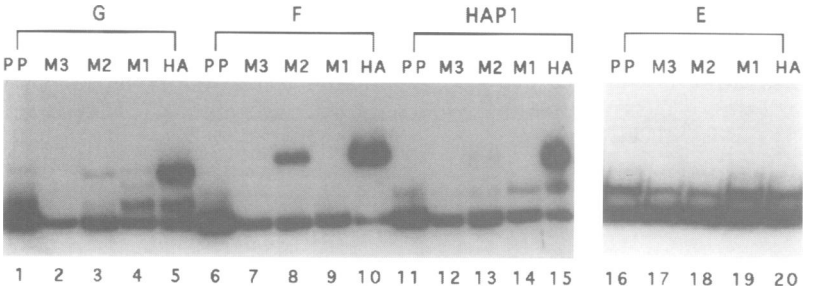

Fig. 3. Comparison of the effects of the binding site mutations on the binding of HAP1 and fusions E, F and G. 50 nM of HAP1 and F, and 100 nM of E and G was incubated with radiolabeled DNA sites: the HAP1 site (HA) (CGGACTTATCGG), mutant M1 (CGGACTTATCCG), mutant M2 (CGGACTCATCGG), mutant M3 (CGGACTCATCCG) or PPR1 site (PP) (CGGCAATTGCCG).

sites. Figure 4C shows that the N3 and TA mutations reduce the affinity of binding. However, in all cases, only the dimeric species was observed, even at low concentrations of protein. Second, we compared dimerization of HAP1 and PPR1 by cross-linking each protein in solution. As shown in Figure 5, the majority of PPR1 (containing residues 29–123) is in dimeric or higher oligomeric forms whereas the majority of HAP1 (containing residues 56–148) is monomeric.

Why is the HAP1 dimerization element able to promote dimerization of HAP1 but not PPR1–HAP1 fusions A and E? This difference can be explained by the interaction between the two zinc clusters of HAP1. This asymmetric interaction not only positions two zinc clusters in a directly repeated orientation, but also helps promote cooperative dimeric binding. In A and E, the asymmetric interaction does not occur.

Discussion

A model for how HAP1 binds to DNA

Here we address one aspect of how the DNA binding domain of the GAL4 family of activators distinguishes among different DNA sites in target genes. In particular, we were interested in how HAP1 binds to direct repeats of CGG while other members of this family bind to inverted repeats. We show that the HAP1 zinc cluster, not the linker, dictates asymmetric HAP1 binding to directly repeated CGGs and that the zinc cluster also helps promote dimerization. Our data suggest a model for the molecular processes involved in HAP1 binding to DNA (Figure 6). In the absence of DNA, the majority of HAP1 is monomeric, and the zinc clusters are apart. When HAP1 binds to DNA, three interactions occur concomitantly: the interaction between the zinc clusters and DNA, the interaction between the two zinc clusters and the interaction between the dimerization elements. These three interactions are all required to lock the complex in a stable conformation.

The critical residues in the HAP1 zinc cluster

What are the residues essential for the asymmetric interaction between the two zinc clusters? In a PPR1–HAP1 fusion A, only the residues before the third Cys are replaced by the corresponding residues of PPR1, but fragment A binds to DNA as a monomer rather than a dimer. This result suggests that A is folded correctly but cannot accommodate the asymmetric interaction necessary for dimeric binding. Further, because residues 65–75 between the first and third Cys are involved in contacting DNA and are physically constrained (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994), we speculate that the N-terminal residues (amino acids 56–64) may be involved in the asymmetric interaction between two zinc clusters. When HAP1 binds to DNA, we imagine that these N-terminal residues of one zinc cluster interact with

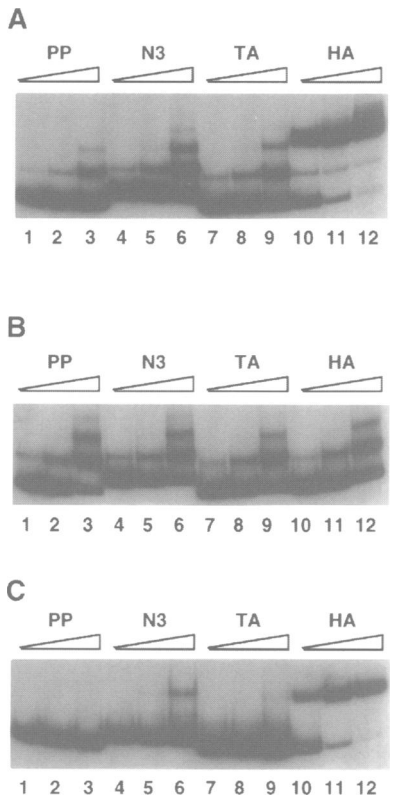

Fig. 4. Dimeric and monomeric binding by HAP1 and fusions E and F. (**A**) HAP1 binding to the HAP1 site (HA, CGGACTTATCGG), the mutant TA site (CGGACTTATATT), the mutant N3 site (CGGACTTATAAACGG) and the PPR1 site (PP, CGGCAATTGCCG). 25 nM (lanes 1, 4, 7 and 10), 50 nM (lanes 2, 5, 8 and 11) and 100 nM (lanes 3, 6, 9 and 12) of HAP1 were incubated with the DNA sites in the DNA binding reactions. (**B**) Fusion E binding to the HA site, the TA site, the N3 site and the PP site. 50 nM (lanes 1, 4, 7 and 10), 100 nM (lanes 2, 5, 8 and 11) and 200 nM (lanes 3, 6, 9 and 12) of fusion E were incubated with the DNA sites in the DNA binding reactions. (**C**) Fusion F binding to the HA site, the TA site, the N3 site and the PP site. 25 nM (lanes 1, 4, 7 and 10), 50 nM (lanes 2, 5, 8 and 11) and 100 nM (lanes 3, 6, 9 and 12) of fusion F were incubated with the DNA sites in the DNA binding reactions. Approximately 0.01 pmol of labeled DNA was used in each reaction.

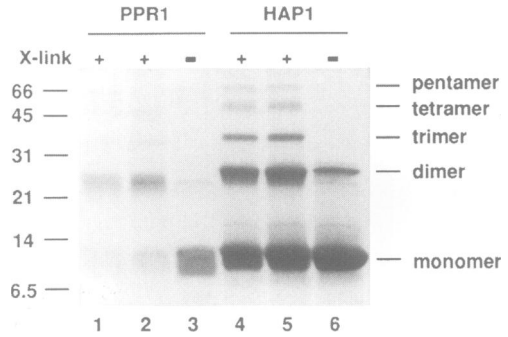

Fig. 5. The HAP1 dimerization element is weaker than that of PPR1. Under conditions for DNA binding reactions, the majority of PPR1 was in dimeric or oligomeric form whereas less than half of HAP1 was in dimeric and oligomeric form (>70% is monomeric). 12 μl solutions containing 0.5 μg (lanes 1 and 4) and 1 μg (lanes 2 and 5) of HAP1 (residues 55–148) or PPR1 (residues 29–123) were treated with 0.002% glutaraldehyde for 1 h prior to loading onto a 15% SDS–polyacrylamide gel. As controls, 1 μg of untreated PPR1 and HAP1 were loaded in lanes 3 and 6, respectively. The gel was subsequently stained with Coomassie brilliant blue, which stains HAP1 much more strongly than PPR1. The amount of proteins was calculated by Bradford assay (Bradford, 1976) and by absorption at 260 nm. The weak bands at the dimer position in both HAP1 and PPR1 may represent a small amount of cross-linked proteins in the preparations.

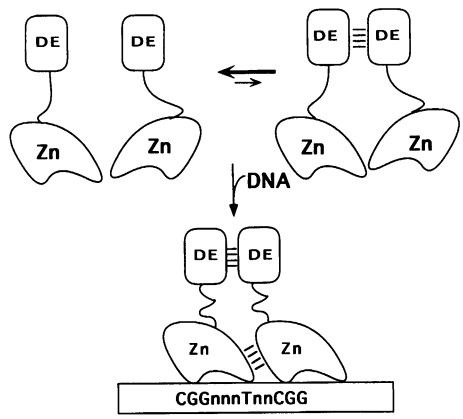

Fig. 6. A model for how HAP1 binds to DNA. In solution, the majority of HAP1 is in the form of monomers. When HAP1 binds to DNA, DNA–HAP1 interactions occur cooperatively with the asymmetric interactions between the two zinc clusters, shown as Zn, and the symmetrical interactions between the dimerization elements, shown as DE, locking the complex in a stable conformation.

the residues on the other side of another zinc cluster (Figure 6), that is, the residues surrounding the sixth Cys. Future NMR or X-ray crystallography analysis should provide details of the structural basis of this asymmetric interaction.

HAP1 asymmetric binding occurs through a novel mechanism

Asymmetric binding has been shown to occur when several other factors, predominantly members of the retinoid receptor family, bind to DNA (Kurokawa *et al.*, 1993; Mader *et al.*, 1993; Perlmann *et al.*, 1993; Towers *et al.*, 1993). Heterodimers and homodimers of retinoid X receptor (RXR), retinoid acid receptor (RAR), thyroid hormone receptor (TR) and vitamin D receptor (VDR) bind to symmetrical DNA sites containing an inverted repeat as well as asymmetrical sites containing a direct repeat (Kurokawa *et al.*, 1993; Mader *et al.*, 1993; Perlmann *et al.*, 1993; Towers *et al.*, 1993). However, this class of factors binds to direct repeats by a mechanism different from that of HAP1 binding. For example, when bound to a direct repeat, the two subunits of the RXR and TR heterodimer are positioned in tandem in a head to tail orientation (Rastinejad *et al.*, 1995). In this heterodimer, residues mediating dimerization and DNA contacts are clustered together; no distinctive dimerization element is present. On the contrary, the C6 zinc cluster proteins, GAL4, PPR1 and HAP1, contain distinctive coiled-coil dimerization elements that only allow symmetrical dimerization (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994; Zhang and Guarente, 1994). To bind to a direct repeat, one of the HAP1 zinc clusters must reorient by 180° (Zhang and Guarente, 1994).

The HAP1 zinc cluster is homologous to that of GAL4 and PPR1 (Johnston, 1987) (45% identity between HAP1 and GAL4). Furthermore, all the key residues in GAL4 or PPR1 known to make base contacts are conserved in HAP1 (outlined residues, Figure 1A) (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). The crystal structures of GAL4 and PPR1 have shown that the C6 zinc clusters adopt well-defined compact structures, and the zinc clusters of GAL4 and PPR1 are virtually superimposable (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994). It is very likely that the HAP1 zinc cluster adopts the same conformation as the zinc clusters of GAL4 and PPR1. Yet, our data clearly show that the HAP1 zinc cluster must also provide an asymmetric interaction between the two zinc clusters to allow asymmetric binding. Therefore, HAP1 provides an example of how a seemingly compact and rigid module can use a novel interaction to expand the repertoire of DNA sequences recognized by a structural motif.

Materials and methods

Construction and purification of HAP1–PPR1 hybrid fragments

The hybrid fragments were constructed by PCRs (oligonucleotide sequence information is available upon request). Amplified DNA fragments were cloned into the T7 expression vector pRSET A (Invitrogen) cut with *Nde*I and *Bam*HI. The plasmids were subjected to DNA sequence analysis to ensure that the correct fusion was made and no mutation occurred. The plasmids were transformed into *Escherichia coli* strain BL21-(DE3)pLysS (Invitrogen), and cells were grown at 37°C in the presence of 50 μg/ml ampicillin until an absorbance of 0.6 at 600 nm was reached. Then, cells were induced with 1 mM IPTG in the presence of 100 μM $ZnOAc_2$. After 2.5 h, the cells were collected by centrifugation. The cells were resuspended in 20 mM HEPES (pH 7.3), 150 mM NaCl, 10% glycerol and 20 μM $ZnOAc_2$ and lysed by passing through a French press. The proteins were then purified to 90% purity from the extract by using an S Sepharose Fast Flow column (Pharmacia) as described (Marmorstein *et al.*, 1992; Marmorstein and Harrison, 1994; Zhang and Guarente, 1994). The amount of proteins was calculated by Bradford assay (Bradford, 1976).

DNA mobility shift assays

The DNA used in DNA mobility shift assays was ~200 bp DNA generated by PCR amplification using single-stranded Bluescript DNA containing a HAP1 site as template. Two M13 sequencing primers, –20 primer (5′-GTAAAACGACGGCCAGTG-3′) and the reverse primer (5′-AACAGCTATGACCATGAT-3′), were used in the PCRs. To end label the DNA, one of the primers was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase prior to PCRs. The amplification was at 94°C, 1 min; 42°C, 2 min; 72°C, 1.5 min for 18 cycles. The labeled DNA was purified on non-denaturing 8% polyacrylamide gels prior to use.

The DNA binding reactions were carried out in 5% glycerol, 4 mM

Tris, pH 8, 40 mM NaCl, 4 mM $MgCl_2$, 10 mM dithiothreitol, 3 μg of salmon sperm DNA, 10 μM $ZnOAc_2$, 300 μg/ml bovine serum albumin. Approximately 0.01 pmol of labeled DNA was used in each reaction. The reaction mixtures were incubated at room temperature for 1 h, and then loaded onto 4% polyacrylamide gels in 1/2TBE for gel electrophoresis at 4°C. Protein concentrations required to bind half of the labeled DNA, which defines the dissociation constant (Fairall *et al.*, 1992), were estimated by carrying out DNA binding reactions with various concentrations of the hybrid proteins.

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