An N-Terminal Dimerization Domain Permits Homeodomain Proteins To Choose Compatible Partners and Initiate Sexual Development in the Mushroom 

Coprinus cinereus

Alison H. Banham,* Rachel N. Asante-Owusu,* Berthold Göttgens,* Sara A. J. Thompson,* Crawford S. Kingsnorth,* E. Jane C. Mellor,b and Lorna A. Casselton a,1

a Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom
b Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom

The A mating-type locus of the mushroom Coprinus cinereus contains three or more paralogous pairs of genes encoding two families of homeodomain proteins (HD1 and HD2). A successful mating brings together different allelic forms of at least one gene, and this is sufficient to trigger initial steps in sexual development. Previous studies have suggested that development is regulated by heterodimerization between HD1 and HD2 proteins. In this report, we describe 5' gene deletions and 5'end exchanges showing that the N-terminal regions of the proteins are essential for choosing a compatible partner but not for regulating gene transcription. Using an in vitro glutathione S-transferase association assay, we demonstrated heterodimerization between HD1 and HD2 proteins and found that heterodimerization only occurs between compatible protein combinations. The N-terminal regions of the proteins were sufficient to mediate dimerization, and N-terminal swaps resulted in a predicted change in dimerization specificity. By analyzing the N-terminal amino acid sequences of HD1 proteins, we identified two potential coiled-coil motifs whose relative positions vary in paralogous proteins but are both required for in vivo function.

INTRODUCTION

Mating in the mushroom Coprinus cinereus commits cells to early events in sexual development; a sterile monokaryon with uninucleate cells is converted to a fertile binucleate-celled dikaryon that differentiates the fruit bodies. Somatic cell fusion initiates mating, but for mating to be successful, the partners must have different alleles of genes at the unlinked A and B mating-type loci (Casselton, 1978). There are an estimated 160 versions of the A locus and 79 of the B locus. Many versions of the genes exist, and their products can only be distinguished as compatible or not by intracellular events following cell fusion. This essential self/nonself-recognition step commits cells to development.

In this report, we focus on the function of the A mating-type genes. The A locus is complex, containing several functionally redundant, multiallelic genes separated into two subcomplexes (a and b) by a region of noncoding DNA (Figure 1). The genes encode a family of homeodomain proteins that are predicted to regulate development by controlling gene transcription. Our molecular analysis of several A loci (Kües et al., 1992, 1994a, 1994b) has shown that the number of genes is variable and that they encode two dissimilar classes of proteins, which we have designated HD1 and HD2 on the basis of conserved but distinct homeodomain sequences (Kües et al., 1992). The A locus has been called a haplotype (May et al., 1991; Kües et al., 1994c), and the archetype is considered to contain four divergently arranged pairs of paralogous HD1 and HD2 genes (Kües and Casselton, 1993). Various parts of the complex are missing in different A loci, as seen for A42 and A6 (Figure 1); each locus has only one complete gene pair (designated the b gene pair) but also solo genes representative of two (A6) or three (A42) other gene pairs (the a, c, and d pairs).

The HD1 and HD2 motifs of the C. cinereus A proteins are conserved in the mating-type proteins of other basidiomycete fungal species. The Aa locus of another mushroom, Schizophyllum commune (Stankis et al., 1992), has a single pair of divergently transcribed HD1 and HD2 genes encoding proteins very similar to those found in C. cinereus, as does the b mating-type locus of two smut fungi, Ustilago maydis and U. hordei (Gillissen et al., 1992; Bakkeren and Kronstad, 1993). The HD1 and HD2 motifs are related to homeodomain sequences in the α1 and α2 mating-type proteins of the budding yeast Saccharomyces cerevisiae (Astell et al., 1984). The S. cerevisiae proteins are known to heterodimerize following mating to generate a functional transcription factor complex that binds target sites upstream of developmentally regulated genes.
Figure 1. Organization of the Wild-Type A42 and A6 Loci of C. cinereus and a Mutant A6 Locus.

The wild-type loci contain several HD7 and HD2 genes separated into the α and β complexes. Arrows indicate direction of transcription. The striped, stippled, black, and white rectangles indicate genes belonging to paralogous gene pairs (α, β, c, or d) and different alleles of the b gene pair. The A6 mutant locus arose by deletion and fusion of the paralogous a2-1 and d1-1 genes.

Herskowitz, 1988; Dranginis, 1990; Goutte and Johnson, 1993; Mak and Johnson, 1993.

Transformation studies using host cells that have genes either experimentally (Gillissen et al., 1992) or naturally deleted (Specht et al., 1992; Kües et al., 1994b) have led to the hypothesis that a similar interaction between HD1 and HD2 proteins might regulate development in the mushroom and smut fungi. Direct evidence has been provided by our analysis of a rare dominant mutation in the A6 locus of C. cinereus that results in a single chimeric gene, part HD2 and part HD1. This gene encodes a fused heterodimer that constitutively promotes A-regulated development. Fusion brings together essential regions of the two proteins, the HD2 homeodomain as the potential DNA binding domain and a predicted activation domain in the C terminus of the HD1 protein. The normal requirement that each protein recognize a compatible dimerization partner has been circumvented, and the chimeric protein is sufficient to promote the A-regulated program of development in the absence of any other A protein (Kües et al., 1994c).

In this study, we investigated the role of the N-terminal domains of the C. cinereus HD1 and HD2 A proteins in regulating gene transcription and in mediating heterodimerization. Our data suggest that this domain is responsible for self/nonself-recognition by permitting heterodimerization only between compatible protein partners.

RESULTS

N-Terminal Domains of HD1 and HD2 Proteins Are Not Required for Regulating Gene Transcription in a Fused Heterodimer

Following cell fusion, the two nuclei derived from each mate remain associated but do not fuse. Their regular distribution in the dikaryotic mycelium is maintained by a complex cell division that results in the formation of a specialized structure known as a clamp connection. Following synchronized division in the tip cell, one of the daughter nuclei must pass through the clamp connection into a uninucleate subterminal cell. The A-regulated pathway of development leads to formation of the clamp cell, but its fusion to the subterminal cell requires activity of the B-regulated pathway (Swiezynski and Day, 1960). When only the A-regulated sequence is active, the clamp cells form but remain unfused; we can use this phenotype to assay
Protein Dimerization Effects Nonself-Recognition

A Constitutive fusion protein

Clamp cells

A6 host

HD2

1 145 387 781

2:61

2:143

B HD2 a2-1 protein

Clamp cells

A5 host

N HD2 C

1 145 220

2:61

2:143

C HD1 b1-1 protein

Clamp cells

A6 host

A5 host

N HD1 C

1 118 594

2:23

2:65

58:99

2:90

Figure 2. Effect of 5' Deletions on the Ability of Genes To Promote Clamp Cell Development.

(A) The constitutive fusion gene.

(B) The HD2 gene a2-1.

(C) The HD1 gene b1-1.

Diagrams indicate the amino acid sequences of the proteins, and numbers indicate (below the bars) the amino acids N-terminal to the homeodomain, the position of the fusion between a2-1 and dl-1 in the mutant, amino acid coordinates of the N-terminal deletions, and the length of each protein. The b1-1 gene used in these experiments lacked an inessential C-terminal 38 amino acids. Graphic symbols used to distinguish the proteins encoded by the different genes are as illustrated in Figure 1. Development of clamps is indicated by a clamp cell.

the function of cloned A genes, following transformation into a suitable host (Mutasa et al., 1990; Kües et al., 1992).

HD1 and HD2 proteins encoded by the paralogous genes in the same locus are normally unable to promote development. However, a rare mutation in the A6 locus has fused the a2-1 gene to the 3' half of the d1-1 gene (Kües et al., 1994c; see Figure 1) and overcome this normal incompatibility by generating a fused heterodimer that constitutively promotes clamp cell development. We used transformation into an A6 wild-type background to demonstrate this constitutive function because there are no potentially compatible A proteins with which this fused heterodimer could interact.

The fusion protein lacks the N-terminal sequence of the HD1 protein (including the homeodomain); thus, this region of the fusion protein cannot be essential for regulating transcription of other genes (Figure 2). By generating 5' deletions of the gene, we were able to show that the N-terminal sequence of the HD2 portion of the fusion protein is also not essential for its constitutive function (Figure 2A). Two versions of the fusion gene were generated by polymerase chain reaction in which the promoter sequences were retained, but sequences encoding amino acids 2 to 62 (pA6md1) or 2 to 143 (pA6md2) were deleted. The latter had only two amino acids in front of the HD2 homeodomain. The complete fusion gene and these two 5' deleted versions were introduced into the A6 test host, and all three were capable of constitutively promoting clamp cell development.

The strategy used to generate the 5' deleted fusion gene was also used to create correspondingly deleted a2-1 HD2 genes. These were also tested for their ability to promote clamp cell development. a2-1 could not promote clamp cell development in the A6 host because it was already present, but it found a compatible partner in a host with an A5 locus that shares none of the genes in A42 and A6 (S.F. O'Shea, E.H. Pardo, and L.A. Casselton, unpublished data). The wild-type gene elicited clamps in this host, but neither of the deleted genes did (Figure 2B). Four 5' truncated versions of the HD1 gene b1-1 were tested also. These were generated by polymerase chain reaction so that the promoter sequence was retained, but sequences encoding amino acids 2 to 23, 2 to 65, 2 to 90, and 58 to 99 were deleted (Figure 2C). b1-1 found a compatible partner in both A5 and A6 hosts, but the truncated genes were inactive in both (Figure 2C). We concluded that the N-terminal regions of the HD1 and HD2 proteins have an essential function no longer required by the fused heterodimer.

N-Terminal Regions of the A Proteins Determine Allele Specificity

Previous studies with genes from A42 (see Figure 1) have implicated the 5' ends in determining gene specificity within the A locus (Kües et al., 1994a). We now asked whether the 5' ends also determine allele specificity (Figure 3). By using conserved restriction sites within the homeodomain-encoding regions of b1-1 and b1-3 and of b2-1 and b2-3, we generated two chimeric genes, namely, b1-3:b1-1 and b2-1:b2-3. The wild-type and chimeric genes were introduced into both A6 and A42 hosts. Wild-type b1-1 and b2-1 elicited clamps in the A6 host, and b1-3 and b2-3 elicited clamps in the A42 host. The chimeric genes in both cases behaved like the gene from which they derived their 5' ends; b1-3:b1-1 behaved like b1-3, and b2-1:b2-3 behaved like b2-1. This result is consistent with the conclusion that the 5' ends of the genes, and hence the N-terminal
Figure 3. Effect of 5' Exchanges on the Mating Specificity of Allelic Versions of the HD1 b1 and HD2 b2 Genes.

Shading is used to distinguish between the two allelic versions of each protein (as given for Figure 1). Numbers indicate the positions at which the amino acid sequences were exchanged.

regions of the proteins, determine allele specificity in addition to gene specificity.

Compatible HD1 and HD2 Proteins Can Heterodimerize in Vitro

Of the genes encoded by the A42 and A6 loci (Figure 1), the only two compatible HD1 and HD2 combinations predicted are b1-1 plus b2-3 and b1-3 plus b2-1. Alleles of paralogous genes are either shared (d1-1 and d1-1) or are inactive (c1-1). Several incompatible protein combinations are present and include b1-1 plus b2-1 or a2-1 and b1-3 plus b2-3 or a2-1. Having implicated the N-terminal regions in determining the specificity of the compatible interaction, we now asked whether these regions mediate protein–protein interactions and whether these interactions are specific.

We have used the in vitro glutathione S-transferase (GST) association assay first developed by Blackwood and Eisenman (1991) to demonstrate dimerization between A proteins. Six different proteins were expressed in Escherichia coli as GST fusion proteins. Following binding to glutathione S-Sepharose beads, the fusion proteins were tested for their ability to bind an in vitro–translated 35S-labeled potential dimerization partner.

In the first experiment, illustrated in Figure 4A, we presented the full-length in vitro-translated HD1 b1-1 protein from A42 with three potential HD2 partners. The HD2 proteins were not full length but contained the entire region N-terminal to the homeodomain, together with varying amounts of C-terminal sequence. The only fusion protein that specifically retained b1-1 was its compatible partner from A6, b2-3. This experiment was repeated using the full-length HD1 b1-3 protein from A6 (Figure 4B), which was specifically retained by its compatible

Figure 4. In Vitro Protein–Protein Interactions in Solution between C. cinereus A Mating-Type Proteins.

Full-length HD1 proteins were 35S-labeled by translation in vitro and adsorbed to a range of GST fusion proteins coupled to glutathione S-Sepharose beads. The lanes contain the following: beads; the GST tag alone (as controls for nonspecific binding); and N-terminal mating-type protein GST fusions. The HD1 proteins are b1-1 (XCT.1, amino acids 1 to 163), b1-3 (pAB97, amino acids 1 to 158), and d1-1 (pAB76, amino acids 1 to 233). The HD2 proteins are a2-1 (pAB79, amino acids 1 to 257), b2-1 (pAB73, amino acids 1 to 166), and b2-3 (pAB47, amino acids 1 to 224).

(A) Interactions with b1-1.
(B) Interactions with b1-3.

Diagrams below the gels represent the in vitro–translated b1-1 and b1-3 proteins, distinguished by stippled and striped shading, respectively, and homeodomain sequences, indicated by a black box. Molecular mass markers are given at the left in kilodaltons (K).
partner from A42 b2-1. A weak interaction was observed between the incompatible b1-3 and b2-3 proteins (only 20% of the binding observed to the compatible partner b2-1). We considered this interaction to be nonspecific because we observed no comparable interaction between the corresponding incompatible b1-1 and b2-1 proteins in the previous experiment. Moreover, the actual sequence mediating this weak interaction (which is also seen, to a lesser extent, in the controls) was at the C terminus of b1-3 and was no longer observed in a subsequent experiment when the protein was truncated (see later text).

In the yeast S. cerevisiae, the a2 protein, which is considered analogous to the C. cinereus HD1 proteins, has a role independent of its compatible partner a1. It homodimerizes via an N-terminal domain (Smith and Johnson, 1992; Vershon and Johnson, 1993) and binds other target sites with another transcription factor, MCM1. We have therefore looked for interactions between our HD1 proteins. Included in the experiments illustrated in Figures 4A and 4B are N-terminal b1-1, b1-3, and d1-1 HD1 GST fusion proteins. We found no evidence to suggest that N-terminal interactions occur in solution between proteins derived from the same HD1 gene (b1-1/b1-1) or from different HD1 genes (b1-1/b1-3 or b1-1/d1-1).

N-Terminal Specificity Is Conferred by the Ability To Form HD1/HD2 Dimers

By exchanging the 5' ends of genes at conserved sites within the region encoding the homeodomain, we showed that allele specificity of both HD1 and HD2 genes in vivo is a function of the N terminus of the proteins (Figure 3). By exchanging the 5' ends of the b1-1 and b1-3 HD1 genes at these same conserved sites, we generated constructs that translated chimeric proteins in vitro and presented these with the same potential HD2 partners as in the previous experiment, that is, a2-1, b2-1, and b2-3. The b1-1:b1-3 protein (Figure 5A) was retained by b2-3, and the b1-3:b1-1 protein (Figure 5B) was retained by b2-1. This result exactly reproduces the change in specificity observed in our in vivo experiment. Noting the positions at which the exchanges were made, we concluded that the regions that determine a compatible interaction lie within the N-terminal 158 amino acids of HD1 proteins. The weak nonspecific interaction of the b1-1:b1-3 protein with b2-1 (only 5.8% of that bound to b2-3) was observed (Figure 5A), and as in the previous experiment, this was attributed to a sequence at the C terminus of b1-3.

N-Terminal Regions of HD1 Proteins Are Sufficient To Mediate Dimerization

Suitable restriction sites in the genes allowed us to translate truncated versions of the b1-1 and b1-3 proteins to determine whether their N-terminal regions were sufficient to mediate specific heterodimerization. As seen in Figures 6A and 6B, the
N-terminal 163 amino acids of b1-1 and 96 amino acids of b1-3 were sufficient to mediate dimerization with their compatible HD2 partners. For the shorter b1-3 fragment, we ruled out involvement of any homeodomain sequences promoting this interaction.

Other predicted helical regions lie immediately C-terminal to the homeodomains in both classes of proteins and at the extreme C terminus of HD1 proteins. It has been proposed that these could also mediate protein–protein interactions (Gieser and May, 1994; Kües et al., 1994a). In a separate experiment (Figure 6C), the C-terminal region of b1-1 (163 to 632 amino acids) was in vitro translated and tested for interactions with full-length HD1-GST fusions and longer HD2-GST fusions. (Full-length HD2 proteins have proved unstable in E. coli, but the expressed proteins contained all helical regions.) The final lane in Figure 6C shows the amount of translated protein presented to the fusion proteins. Unlike the compatible interactions observed in Figures 4 and 5, where consistently more than 50% of the in vitro–translated protein was retained on the beads, only a weak interaction (at most, 9%) was observed between b1-1 and the GST proteins, and this occurred to varying extents with all of them. We concluded that the C-terminal regions of the proteins are unlikely to mediate specific dimerizations in solution. Our overall conclusion from these in vitro experiments is that specific interactions in solution are only possible between compatible proteins.

Predicted Motifs within the N-Terminal Domains

Using the algorithm derived by Lupas et al. (1991), Gieser and May (1994) predicted the presence of two coiled coils within the N-terminal region of two allelic HD1 C. cinereus A proteins.

### Table 1. Coiled-Coil Motifs Predicted in HD1 Mating-Type Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>First Coil</th>
<th>Second Coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino</td>
<td>Score*</td>
<td>Amino</td>
</tr>
<tr>
<td>Acids</td>
<td></td>
<td>Acids</td>
</tr>
<tr>
<td>b1-1</td>
<td>15 to 33</td>
<td>1.49 to 1.62</td>
</tr>
<tr>
<td>b1-3</td>
<td>18 to 33</td>
<td>1.16</td>
</tr>
<tr>
<td>b2-1</td>
<td>24 to 33</td>
<td>1.41 to 1.61</td>
</tr>
<tr>
<td>d1-1</td>
<td>37 to 59</td>
<td>1.34 to 1.51</td>
</tr>
<tr>
<td>a2</td>
<td>16 to 29</td>
<td>1.48</td>
</tr>
</tbody>
</table>

Sequence analysis was performed using the method described by Lupas et al. (1991). In this method, amino acid sequences are analyzed in terms of empirically determined residue preferences for the seven positions (a through g) in a coiled-coil heptad repeat.

* The score is related to the probability of the occurrence of a coiled coil at a given position in the sequence. A score of 1.3 or above indicates a significant probability of coil formation.
DISCUSSION

Sexual development in *C. cinereus*, as in *S. cerevisiae*, is regulated by two dissimilar classes of homeodomain proteins. Heterodimerization between the a1 and a2 proteins determines the developmental fate of mated *S. cerevisiae* cells by generating a diploid cell—specific transcription-factor complex. In this paper, we present evidence that development is similarly regulated by heterodimerization between the HD1 and HD2 homeodomain proteins encoded by the A mating-type locus of *C. cinereus*. Our *in vitro* studies indicate that HD1 and HD2 homeodomain proteins are able to heterodimerize in solution via N-terminal domains that have predicted structural similarity to corresponding dimerization domains in the a1 and a2 proteins.

Unlike *S. cerevisiae*, the basidiomycete fungi have evolved multiple mating types (Kües and Casselton, 1992). In *C. cinereus*, this has been achieved by having several multiallelic, paralogous genes at each mating-type locus. An unmated cell has several functionally equivalent HD1 and HD2 proteins that must be prevented from promoting development. These same proteins, however, must be able to interact successfully with other versions of the proteins following mating. From the results of the experiments described in this study, we suggest that incompatible protein partners are distinguished from compatible ones in that they are unable to heterodimerize. We used a GST association assay in which we presented the b1-1 protein, encoded by an HD1 gene in the A42 locus, with two incompatible HD2 partners encoded by genes at the same locus, a2-1 and b2-1, and a compatible partner encoded by a different allele of b2 at the A6 locus, b2-3. Only the compatible protein pair, b1-1 and b2-3, dimerized. We showed, similarly, that the HD1 protein b1-3 encoded by the A6 b1 gene only dimerized with its compatible partner b2-1 from A42.

By truncating the proteins used in the *in vitro* assay, we identified sequences N-terminal to the homeodomain as those mediating dimerization. These regions of both protein classes were implicated in determining the specificity of a compatible protein interaction in vivo by exchanging the 5' ends of both paralogous genes (Kües et al., 1994a) and alleles of the same gene (this study). In all cases, chimeric genes had the mating-type specificity of the gene from which they derived their 5' ends and thus the N-terminal domains of the proteins. The results of these in vivo experiments were the same as the results of *in vitro* experiments with similarly derived chimeric proteins. We exploited the unique properties of the A6 mutant fusion gene to demonstrate, using 5' gene deletions, that these normally essential specificity domains of both classes of proteins are not necessary for A-regulated transcription of other genes. We suggest that their major role is to allow HD1 and HD2 proteins to choose compatible partners by dimerization.

The Lupas et al. (1991) COILS program predicts two coiled-coil motifs in the N-terminal domains of HD1 proteins (Gieser and May, 1994; Table 1). Several potential coiled coils in the N-terminal regions of the HD2 proteins with which the HD1 coils could interact are also predicted by this analysis, but their positions are not so clearly defined. N-terminal coils are also predicted in the analogous HD1 proteins of *S. cerevisiae* and the bE proteins of *U. maydis* (data not shown), suggesting that these are conserved features of basidiomycete HD1 mating-type proteins. These motifs are found in the corresponding N-terminal regions of both a2 and a1 of *S. cerevisiae* (Ho et al., 1994) and have been implicated in dimerization by mutation. Deletions and amino acid substitutions in the d positions of the 3,4-hydrophobic heptad repeat sequences of a2 protein disrupted heterodimer stability in vitro and reduced a1/a2 activity in vivo (Ho et al., 1994).

The N-terminal amino acid sequences of different *C. cinereus* HD1 and HD2 proteins are highly variable (Kües et al., 1994a), and the dimerization potential of proteins is undoubtedly influenced by different amino acids. Currently, we do not have sufficient protein sequences to make useful predictions about why, for example, b2-1 can heterodimerize with several allelic variants of b1 but cannot dimerize with its allelic partner in A42, b1-1. Failure to dimerize would appear to be a more specific interaction than the ability to dimerize. The e and g positions that flank the hydrophobic a and d positions of the 3,4-heptad repeat often contain charged amino acids that are thought to interact electrostatically (Cohen and Parry, 1990; Baxevanis and Vinson, 1993), and comparisons of dimer stabilities of other proteins that interact by means of coiled coils, such as Fos, Jun, and GCN4, suggest that it is the amino acids at these positions that determine the specificity of the interactions (O'Shea et al., 1992; Glover and Harrison, 1995). With so many genes encoding the HD1 and HD2 A proteins of *C. cinereus*, there may be more than one constraint on dimerization, and the actual positions of the N-terminal coils may be one of these. Although the second predicted coil in all four HD1 proteins we examined was at the same position (amino acids 37 to 59), the first coil in the paralogous d1-1 protein was displaced (amino acids 37 to 59) relative to that in the three allelic versions of b1 (amino acids 14 to 33). It would have been
interesting to look for this coil in other α1 proteins, but so far we have only identified this one allele in our stock collection.

The entire region N-terminal to the homeodomain of both HD1 and HD2 proteins is essential for a normal compatible interaction in vivo, because none of the 5' deleted genes had function in vivo. Deletions of amino acids 2 to 65 would have removed the first predicted coil (amino acids 14 to 33) in the HD1 b1-1 proteins, and deletions of amino acids 58 to 99 would have removed the second (73 to 99). Interestingly, deletions of one or the other predicted coil from the α2 protein of S. cerevisiae only reduced the stability of the heterodimer (Ho et al., 1994). With the complex function of discriminating incompatible protein partners, dimerization through this region may have acquired a more essential function for the C. cinereus proteins.

Significance of Heterodimerization of Homeodomain Proteins

The homeodomain sequence is very conserved, and choosing a dimerization partner for cooperative DNA binding can confer specificity for target site selection. In the case of the α2 protein of S. cerevisiae, association with either α1 or MCM1 imposes different spacing of otherwise similar homeodomain contact sites on DNA (Smith and Johnson, 1992) and leads to recognition of different targets and regulation of different sets of genes. Sequences adjacent to the homeodomains are critical in these protein–protein interactions, a C-terminal dimerization domain for α1/α2 and a sequence immediately N-terminal to the homeodomain for α2/MCM1, indicating that conformational effects may be important (Goutte and Johnson, 1993, 1994; Mak and Johnson, 1993; Stark and Johnson, 1994). Such interactions are not confined to fungal homeodomain proteins, and similar examples can be found in Drosophila, mammals, and Caenorhabditis (see reviews in Manak and Scott, 1993; White, 1994). The N-terminal dimerization domain in the C. cinereus A proteins is not required to promote target site selection because we have shown it to be dispensable in the chimeric fusion gene product. We have suggested that a conserved α-helical domain in both proteins just C-terminal to the homeodomains may be analogous to the C-terminal dimerization domain in the S. cerevisiae proteins (Kües et al., 1994a). If this C-terminal dimerization were DNA dependent, as it is with α1 and α2 (Dranginis, 1990; Mak and Johnson, 1993), we would have been unable to detect it in our in vitro assay in the absence of DNA.

The N-terminal domain of the mating-type proteins may be more analogous to an N-terminal domain of the POU-like homeodomain protein HNF-1, which also mediates dimerization in solution. This is thought to allow HNF-1 to choose different proteins as dimerization partners to diversify the activities associated with a single target site (Mendel et al., 1991). In the proteins we studied, choosing a dimerization partner is not required to diversify function or to select a different target site but to act as a mechanism that allows cells to distinguish self from nonself.

METHODS

Coprinus cinereus Strains

Strains used as transformation hosts were the trypophan auxotrophs LNI118 (A42B42 ade-2 trp-1.1,16), LT2 (A6B6 trp-1.1,16), and FA2222 (A6B5 trp-1.1,16). Plasmids containing A mating-type genes were cotransformed with plasmid pCcl001 (Binninger et al., 1987) containing the C. cinereus trp-1 gene. At least 50 trp+ transformants from each experiment were screened microscopically for the presence of clamp cells. Media and general methods of culture were those described by Lewis (1961) and Mutasa et al. (1990). The transformation procedure was based on Casselton and de la Fuente Herce (1989).

Plasmid Constructs Used for in Vivo Studies

pBluescript KS− clones containing genes used in C. cinereus transformations were pAMT1 (complete b1-1 sequence) and pAMT2 and pAMT3 (a 3' truncated version of b1-1 lacking the terminal 120 bp), as described by Tymon et al. (1992), pESM1 and pUK4 (b2-1), pESM2 (4-1), and pUK2 (a2-1) are described by Kües et al. (1992), pA625 and pA626 contain the b1-3 and b2-3 genes on 2.2- and 5.5-1b XhoI fragments, respectively. pWRFl contains the A6 fusion gene (Kües et al., 1994c). Chimeric gene b1-3-1 was constructed using a conserved C-terminal site within the homeobox sequences of b1-1 (in pAMT3) and b1-3 (in pA626) to give pB1-3-1. Chimeric gene b2-3-1/3 was constructed using a conserved NcoI site immediately 3' to the homoeo domain coding sequence using b2-1 (in pUK4) and b2-3 on a 1.9-kb XhoI-SacI fragment derived from pA625 to give pB2-1/3. 5' Gene deletions were generated by polymerase chain reaction. Primers used to truncate a2-1 (in pUK2) were 5'-CATGGTGAAAGCGATGTGGAGG-3' with either 5'-CTCCACACTGTCCTCCG-3' or 5'-TACGTGCCCCTCATCAAG-3' to delete nucleotides 4 to 183 and 4 to 429, respectively. Primers used to truncate b1-1 (in pAMT2) were 5'-CTCCAGCCTAGTGCCTCC-3' with 5'-ACGCTGGCGCGGATCTGCTG-3' deleting nucleotides 4 to 66), with 5'-CTCCAGCCTAGTGCCTCGTC-3' (deleting nucleotides 4 to 192), and with 5'-GCGGATTGGAGAGAGGAGG-3' and 5'-GCGGATTTCCCAAGACAGC-3' were used to delete nucleotides 153 to 299. NhoI-SalI fragments encompassing the deletions in a2-1 were subcloned for sequencing and then used to replace the appropriate nondeleted sequence in pUK2 (to give pA2d1 and pA2d2) and the a2-1 sequence of the A6 fusion gene in pWRFl (to give pA6d1 and pA6d2). HindIII-EcoRV fragments encompassing the deletions in b1-1 were similarly subcloned for sequencing and used to replace the appropriate nondeleted sequence in pAMT2.

Plasmid Constructs Used for in Vitro Assays

cDNAs for A42 genes were derived from a λgt10 library (Mutasa et al., 1990), and cDNAs for A6 genes were derived from a newly constructed library in λZAP II (Stratagene). Table 2 lists all plasmids
Table 2. Plasmid Constructs for Protein Expression Used in This Study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>cDNA</th>
<th>Amino Acids</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAB52</td>
<td>b1-1</td>
<td>1 to 632</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB109</td>
<td>b1-1</td>
<td>1 to 163</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB81</td>
<td>b1-1</td>
<td>163 to 632</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB94</td>
<td>b1-1</td>
<td>1 to 163:159 to 630</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB93</td>
<td>b1-3</td>
<td>1 to 630</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB95</td>
<td>b1-3</td>
<td>1 to 96</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB88</td>
<td>b1-3</td>
<td>1 to 158:164 to 632</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB72</td>
<td>d1-1</td>
<td>1 to 632</td>
<td>pTM1</td>
</tr>
<tr>
<td>pAB56</td>
<td>b1-1</td>
<td>1 to 632</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pXCT.1</td>
<td>b1-1</td>
<td>1 to 163</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pAB100</td>
<td>b1-3</td>
<td>1 to 630</td>
<td>pGEX-3X</td>
</tr>
<tr>
<td>pAB97</td>
<td>b1-3</td>
<td>1 to 158</td>
<td>pGEX-3X</td>
</tr>
<tr>
<td>pAB70</td>
<td>d1-1</td>
<td>1 to 632</td>
<td>pGEX-1</td>
</tr>
<tr>
<td>pAB76</td>
<td>d1-1</td>
<td>1 to 233</td>
<td>pGEX-1</td>
</tr>
<tr>
<td>pAB78</td>
<td>a2-1</td>
<td>1 to 358</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pAB79</td>
<td>a2-1</td>
<td>1 to 257</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pAB2-EX.2</td>
<td>b2-1</td>
<td>59 to 320</td>
<td>pGEX-3X</td>
</tr>
<tr>
<td>pAR73</td>
<td>b2-1</td>
<td>1 to 166</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pAB87</td>
<td>b2-3</td>
<td>1 to 248</td>
<td>pGEX-2T</td>
</tr>
<tr>
<td>pAB47</td>
<td>b2-3</td>
<td>1 to 224</td>
<td>pGEX-2T</td>
</tr>
</tbody>
</table>

cDNA sequences encoding fragments of the A proteins were cloned into the vector pTM1 for translation in vitro and into pGEX vectors for expression in E. coli. The amino acid numbers refer to the region of the protein translated, starting with 1 as the N-terminal methionine. A colon indicates a chimeric cDNA construct and corresponding amino acid sequences contributed by each gene.

generated for protein expression in Escherichia coli and for in vitro transcription and translation.

T7 in Vitro Transcription and Translation of Proteins

T7 expression plasmid DNA (1 to 2 μg) was transcribed and translated in vitro using a TNT T7 coupled reticulocyte lysate system (Promega), labeling with 35S-methionine, according to the manufacturer's instructions. cDNAs for T7 expression were cloned into the T7 expression vector pTM1 (Elroy-Stein et al., 1989).

Expression of Glutathione S-Transferase Fusion Proteins and Preparation of Extracts

cDNAs for expression were cloned into pGEX vectors (Pharmacia Biotech Ltd.) and transformed into E. coli BL21 (DE3) (Studier et al., 1990). Cells were grown overnight at 37°C in Luria-Bertani medium plus 100 μg/mL ampicillin. Twenty milliliters of the overnight culture was used to inoculate 200 mL of Luria-Bertani medium plus 200 μg/mL ampicillin. After shaking at 37°C for 1 hr, expression of the fusion protein was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. After continuing the incubation for either 3 hr (pGEX, XCT.1, pAB97, pAB76, pAB79, pAB47, pAB78, pAB2-1 Ex.2, and pAB87) or 6 hr (pAB53, pAB56, pAB100, and pAB70), cells were pelleted and washed with 20 mL of PBS; the cell pellet was then frozen at −70°C for 1 hr. The cells were defrosted, resuspended in 20 mL of MTPBS (150 mM NaCl, 16 mM Na2HPO4·7H2O, 4 mM NaH2PO4, pH 7.3), and then lysed by sonication. Cell debris was removed by centrifugation, and the supernatant was aliquoted in 1-mL volumes and stored at −70°C. The presence of the fusion protein was confirmed by SDS-PAGE.

pGEX Dimerization Assay

Using this in vitro technique, protein–protein interactions were identified by the ability of a glutathione S-transferase (GST) fusion protein bound to Sepharose beads (Pharmacia Biotech Ltd.) to retain a labeled partner through a series of wash steps. The dimerization assay was based on Zappavigna et al. (1994). Twenty-microtiter aliquots of glutathione S-Sepharose beads (Pharmacia Biotech Ltd.) were washed three times with 1 mL MTPBS. One-milliliter aliquots of the appropriate GST fusion protein were bound to the beads by incubation at room temperature for 10 min, then 10 min on ice. The beads were washed twice with 1 mL of MTPBS and once with 1 mL of HND buffer (20 mM Hepes, pH 7.2, 150 mM NaCl, 5 mM DTT, 0.1% Nonidet P-40, 10 mg/mL BSA). Washed beads were resuspended in 200 μL HND buffer; after 5 min on ice, 5 μL of the in vitro–translated protein from a single reaction was added to each tube of beads. After incubating at 4°C and being gently shaken for 1 hr, the beads were washed four times with 1 mL of MTPBS containing 0.1% Nonidet P-40. The beads were resuspended in 50 μL of Laemmli sample buffer, and 20 μL of each sample was run on an SDS–polyacrylamide gel. The gel was fixed for 30 min (10% methanol, 10% acetic acid), amplified for 15 min (Ampify, NAMP 100; Amersham), dried, and autoradiographed.

**ACKNOWLEDGMENTS**

We thank Ian Connerton for help with the PCR strategies used to truncate genes, Mark Sansom for valuable help with the COiL computer analysis, Liz Cowe for helping us run the program, and Michael Milner for isolating and helping to sequence the b-3 cDNA. This work was supported by Biotechnology and Biological Sciences Research Council Grant Nos. PG04W0564 and P02352 to L.A.C. and E.J.C.M., a research studentship to R.N.A.-O., and a postdoctoral fellowship to L.A.C. B.G. received support from a Science and Engineering Research Council studentship and the Gatsby Charitable Foundation. E.J.C.M. is a Wellcome Trust Senior Research Fellow.

Received February 22, 1995; accepted April 12, 1995.

**REFERENCES**


An N-Terminal Dimerization Domain Permits Homeodomain Proteins To Choose Compatible Partners and Initiate Sexual Development in the Mushroom Coprinus cinereus.
A H Banham, R N Asante-Owusu, B Gottgens, S Thompson, C S Kingsnorth, E Mellor and L A Casselton

*Plant Cell* 1995;7:773-783
DOI 10.1105/tpc.7.6.773

This information is current as of November 6, 2017

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>eTOCs</td>
<td>Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>CiteTrack Alerts</td>
<td>Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>Subscription Info</td>
<td>Subscription Information for <em>The Plant Cell</em> and <em>Plant Physiology</em> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a></td>
</tr>
</tbody>
</table>