The EMBO Journal vol.13 no.17 pp.4054–4059, 1994

A chimeric homeodomain protein causes self-compatibility and constitutive sexual development in the mushroom *Coprinus cinereus*

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Communicated by C.J.Leaver

The *A* mating type genes of the mushroom *Coprinus cinereus* encode two classes of putative transcription factor with distinctive homeodomain motifs (HD1 and HD2). A successful mating brings together different allelic forms of these genes and this triggers part of a developmental sequence required for sexual reproduction. In this report we provide evidence that this developmental programme is promoted by a physical interaction between the two classes of homeodomain protein. Rare dominant mutations conferring self-compatibility map to the *A* locus and result in constitutive operation of the *A*-regulated developmental pathway. Our molecular analysis of one of these mutations shows that it has generated a chimeric gene by in-frame fusion of an HD2 and an HD1 gene. Fusion has overcome the normal incompatibility between two proteins coded by genes of the same *A* locus and generated a protein that is sufficient to promote development in the absence of any other active *A* mating type genes. The fusion protein retains most of the HD2 sequence, but only the C-terminal part of the HD1 protein. It has only the HD2 homeodomain motif as a potential DNA binding domain fused to an essential C-terminal region of the HD1 protein, which in a normal HD1–HD2 protein complex may be the major activation domain.

Key words: *A* mating type proteins/chimeric homeodomain protein/*Coprinus*/'fused dimer'/self-compatibility

Introduction

Self-incompatibility is a common rule throughout sexually reproducing species and genetic mechanisms that have evolved to prevent selfing rarely break down. In the fungi, where sexual dimorphism is uncommon, self-incompatibility is imposed by the mating type genes. The mushroom fungi are particularly interesting, because they have multiple mating types; in *Coprinus cinereus*, it is estimated that there may be as many as 12 000 (Whitehouse, 1949; Raper, 1966). These are generated by multi-allelic genes at two unlinked loci, classically known as the *A* and *B* mating type factors (Raper, 1966), which independently regulate different steps in a developmental pathway that initiates sexual reproduction. When the *A* and *B* mating type genes are different, fusion of two asexual mycelia (monokaryons) with uninucleate cells results in the formation of a fertile binucleate celled mycelium (dikaryon) on which the fruit bodies develop (Swiezynski and Day, 1960). Mutational studies, designed to understand how extensive allelic variation at the *A* and *B* loci is generated, unexpectedly led to the isolation of rare dominant mutations conferring self-compatibility. Such mutations have been isolated in both *C.cinereus* and in another mushroom, *Schizophyllum commune*, and map within one or other of the mating type loci (Day, 1963; Parag, 1962; Raper *et al.*, 1965; Haylock *et al.*, 1980). In this study we describe the molecular basis of a mutation within the *A* locus of *C.cinereus*.

The *A* locus is a complex of genes which are separated into two subcomplexes, known classically as the α and β loci (Day, 1960). The genes encode a homeobox-containing family of proteins, divisible into two classes, HD1 and HD2, on the basis of their conserved but different homeodomain sequences (Kües *et al.*, 1992). The different *A* factors, perhaps best called haplotypes (May *et al.*, 1991), exhibit much structural variation. The archetypal form is considered (Kües and Casselton, 1993) to contain four divergently arranged HD1–HD2 gene pairs, but different parts of the complex are missing in different haplotypes. Factor *A6* retains only one intact HD1–HD2 pair and two solo genes, one of each class (Figure 2). Apart from their HD motifs, all the genes are highly divergent and polymorphic, both between and within haplotypes, and show little or no cross-hybridization.

Previous analysis, involving gene cloning and transformation, has led to the hypothesis (Kües and Casselton, 1993; Kües *et al.*, 1994) that *A*-regulated development depends on the interaction (presumably dimerization) of certain pairs of HD1 and HD2 proteins encoded in different haplotypes and that, for as yet unknown reasons, active dimers are not formed by HD1 and HD2 products of the same haplotype. The self-compatible mutation described here occurred within the *A6* locus. Fusion between normally incompatible HD1 and HD2 genes has generated a gene that can be translated to give a chimeric protein that constitutively activates sexual development.

Results

Cloning the self-compatible A6 locus

The dikaryon of *C.cinereus* has binucleate cells, one nucleus derived from each monokaryotic mate, and these are distributed to each daughter cell by a complex division that involves the formation of a specialized structure known as the clamp connection (Casselton, 1978). Following nuclear division in the apical cell, one of the nuclei

must pass through the clamp cell into the subapical cell. The pairing of compatible *A* factors promotes the formation of the clamp cell and pairing of different *B* factors promotes its fusion with the subapical cell. When only the *A*-regulated part of sexual development is operating, clamp cells are formed but cannot fuse and the nucleus that is in the clamp becomes trapped. Mutations in the *A* locus that confer a self-compatible *A* mating type also lead to development of unfused clamp cells, as shown in Figure 1 (Day, 1963), and thus cause constitutive expression of the *A*-regulated developmental pathway.

The mutant illustrated was isolated by Day from a wild-type *A6* strain. We used genomic DNA from this mutant strain to construct a cosmid library and a single clone containing the *A6* mutant locus was identified by hybridization using common flanking sequences from another cloned *A* locus (Mutasa *et al.*, 1990; Kües *et al.*, 1992). Transforming DNA generally integrates non-homologously in *C.cinereus*, therefore a transformed host will express both the resident and introduced *A* genes. The mutant sequence isolated was able to promote clamp cell development in two host strains having non-mutant *A* loci, one with the *A5* haplotype and one with *A6*. *A5* was chosen because it is known from hybridization data that it does not share any specificity genes with *A6* (Kües *et al.*, 1992; unpublished observations). The wild-type *A6* locus contains four specificity genes (designated *a2-1, b1-3, b2-3* and *d1-1*) (Figure 2) and all four were shown to individually elicit clamp cell development when introduced into the *A5* host; none could do so in the *A6* self background. In the *A5* host a normal compatible interaction between proteins coded for by genes from the mutant locus and the *A5* wild-type may be expected, but only the mutant self-compatible *A6* will be able to promote clamp cell development in the *A6* host.

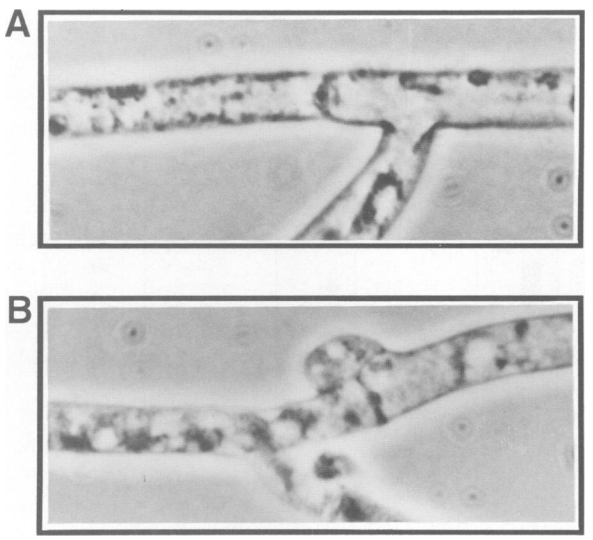

Fig. 1. Effect of a mutation in the *A6* mating type locus on hyphal morphology. (**A**) The wild-type monokaryon has simple septa, whereas (**B**) the *A6* self-compatible mutant produces unfused clamp cells.

Self-incompatibility is associated with a major deletion

A 4.5 kb *Eco*RI fragment from the cosmid clone containing the mutant *A6* locus (pWFR1) proved to be sufficient to elicit the dominant self-compatible phenotype in the wild-type *A6* host. This fragment hybridized to two genes in the wild-type, *a2-1* and *d1-1* (data not shown). These two genes are normally 12 kb apart, thus a major DNA rearrangement must have occurred to generate the mutation. By comparing the restriction maps of the wild-type and mutant it was evident that the entire region between *a2-1* and *d1-1* was missing in the mutant (Figure 2). Hybridizations to genomic DNA confirmed that these sequences had been lost and not translocated (Figure 3).

Gene fusion generates a chimeric homeodomain protein

The 4.5 kb *Eco*RI fragment containing the mutant *A6* function identified three transcripts in mRNA from a wild-type *A6* strain. These corresponded to *a2-1*, *d1-1* and the small β-flanking gene (Figure 4A), whose transcript overlaps that of *d1-1* (Kües *et al.*, 1992). In mRNA derived from the self-compatible *A6* mutant, the *a2-1* and *d1-1* transcripts were replaced by a larger 3.2 kb transcript. This new transcript hybridized to the *a2-1* gene and to

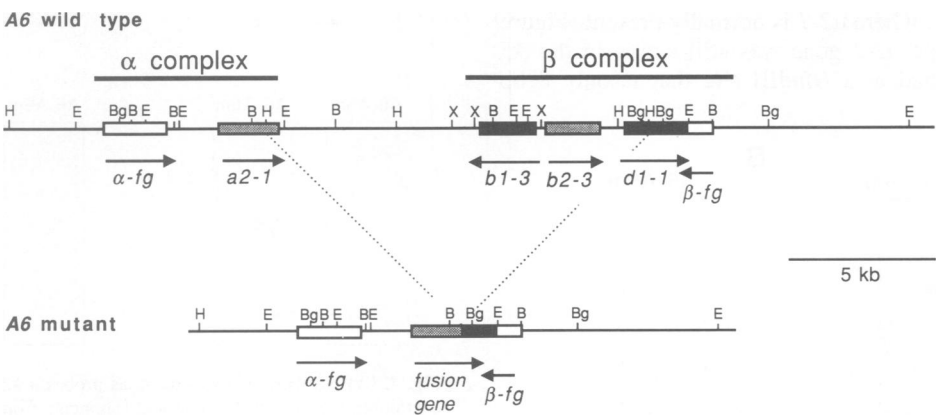

Fig. 2. Comparative restriction maps of the wild-type and self-compatible *A6* mating type loci showing genes and direction of transcription (arrows) determined by sequencing and selective strand labelling in Northern blots. Genes of the wild-type are separated into two complexes α and β by ~7.0 kb of non-coding sequence. There are four specificity genes; *a2-1* and *b2-3* are HD2 genes (grey boxes) and *b1-3* and *d1-1* are HD1 genes (black boxes). α-*fg* and β-*fg* (open boxes) are non-specific genes found flanking all *A* loci (Kües *et al.*, 1992; Richardson *et al.*, 1993). Gene designations follow the classification of Kües and Casselton (1993). Restriction sites are represented by: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I.

the 3′ end of the *d1-1* gene (Figure 4A). *a2-1* and *d1-1* are normally transcribed in the same direction (Kües *et al.*, 1992). By using strand-specific probes, it was confirmed that this was also true in the mutant (Figure 4B). The *a2-1* and *d1-1* genes found in the *A6* haplotype are also present in the *A42* haplotype described by Kües *et al.* (1992) and the complete DNA sequences of these genes are known [EMBL data bank, accession numbers X79686 (CCA21) and X79688 (CCD11)]. Sequencing across the point of fusion in the mutant confirmed that the genes are transcribed in the same direction and demonstrated that the coding sequences have been fused in the same reading frame (Figure 5). The actual fusion point occurs within a region where there is a 4 bp homology between *a2-1* and *d1-1*. There are also some incomplete homologies in the surrounding sequence that may have facilitated the recombination event.

Translation of the fused coding sequences should yield a chimeric protein containing the first 387 amino acids of the HD2 *a2-1* protein fused to the C-terminal 394 amino acids of the HD1 *d1-1* protein. The full-length *a2-1* and *d1-1* proteins are 520 and 632 amino acids respectively; the chimeric protein has 781 amino acids. The most striking feature of the mutant protein is that it has only a single homeodomain and this is derived from the HD2 protein (amino acids 147–206).

3′ Truncation of the fusion gene restores a self-incompatible phenotype

Figure 6A shows the position of the mutant fusion gene within the cloned 4.5 kb *Eco*RI fragment and the relative sequences derived from *a2-1* and *d1-1*. The entire gene is also present in the smaller *Dra*I−*Eco*RI fragment. Both these fragments were equally capable of eliciting *A*-regulated clamp cell development in wild-type *A6* and *A5* host strains. Cutting the *Eco*RI fragment with *Bam*HI yielded two fragments that were inactive in both host strains. One fragment contains the 3′ end of *a2-1* and *d1-1* but lacks any 5′ promoter sequences. The other fragment contains the promoter and 1.3 kb of the 5′ end of *a2-1*, which is obviously too short to retain function. The wild-type *a2-1* gene can promote clamp cell development in an *A5* host which contains a compatible HD1 gene, but not in the *A6* host, where *a2-1* is normally present (Figure 6B). The wild-type *a2-1* gene was still active in the *A5* host when truncated at a *Hin*dIII site that is only 8 bp downstream of the fusion point in the mutant gene (Figure 5). By cutting the mutant gene as close as possible to the fusion point (398 bp downstream) using a *Bgl*II site in the *d1-1* sequence (Figure 2), a normal *a2-1* function was recovered, but the self-compatible constitutive function was lost. This fragment promoted clamp cell development in the *A5* host but not in the *A6* host and thus behaves as a wild-type *a2-1* gene.

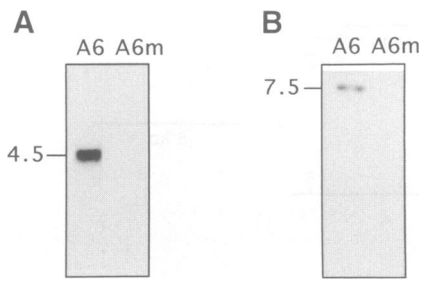

Fig. 3. Southern blot analysis of genomic DNA from a wild-type and mutant *A6* strains showing that ~12 kb of genomic sequence is missing in the mutant. Probes were (**A**) a 4.5 kb *Hin*dIII fragment from the non-coding region separating the α and β complexes and (**B**) a 7.5 kb *Hin*dIII fragment containing the *b1-3* and *b2-3* specificity genes.

Discussion

Self-compatible mutations within the *A* factor of *C.cinereus* are so rare that only a very strong positive

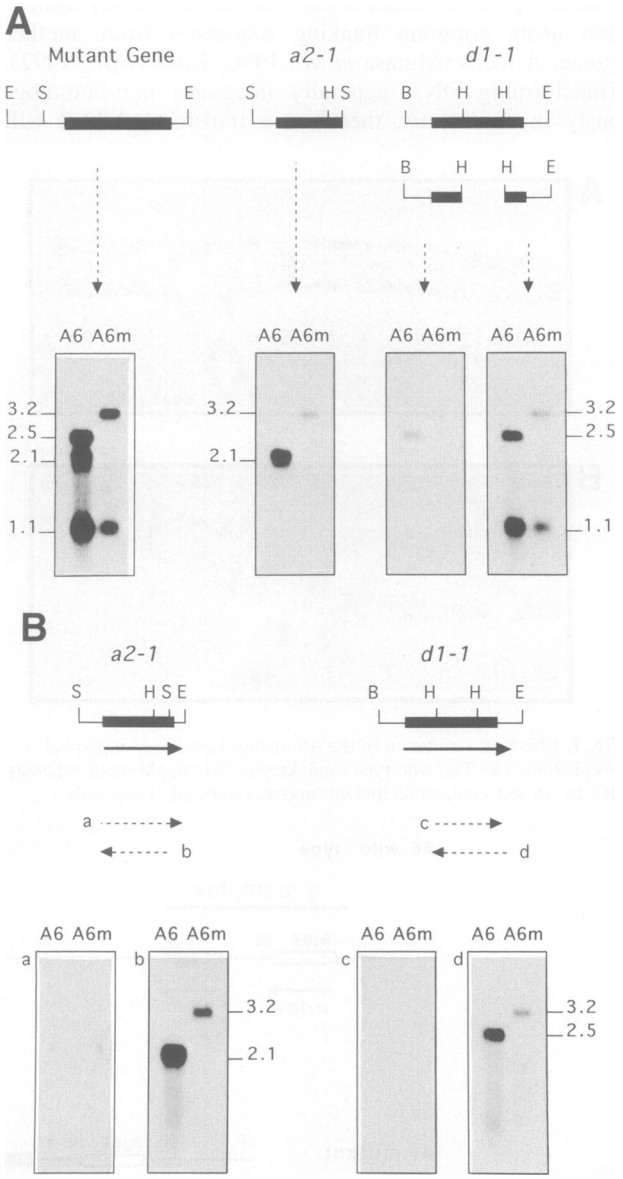

Fig. 4. (**A**) Northern analysis using as probes a 4.5 kb *Eco*RI fragment containing the mutant *A6* gene and fragments from the wild-type containing *a2-1*, *d1-1* and the separated 5′ and the 3′ ends of *d1-1*. The 3′ end of the *d1-1* gene overlaps that of the β-*fg* from which the 1.1 kb transcript derives. (**B**) Northern analysis to demonstrate that *a2-1*, *d1-1* and the mutant fusion gene are all transcribed in the same direction. Strand-specific probes (dashed arrows) were derived from *a2-1* and *d1-1*. Restriction sites are shown as: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

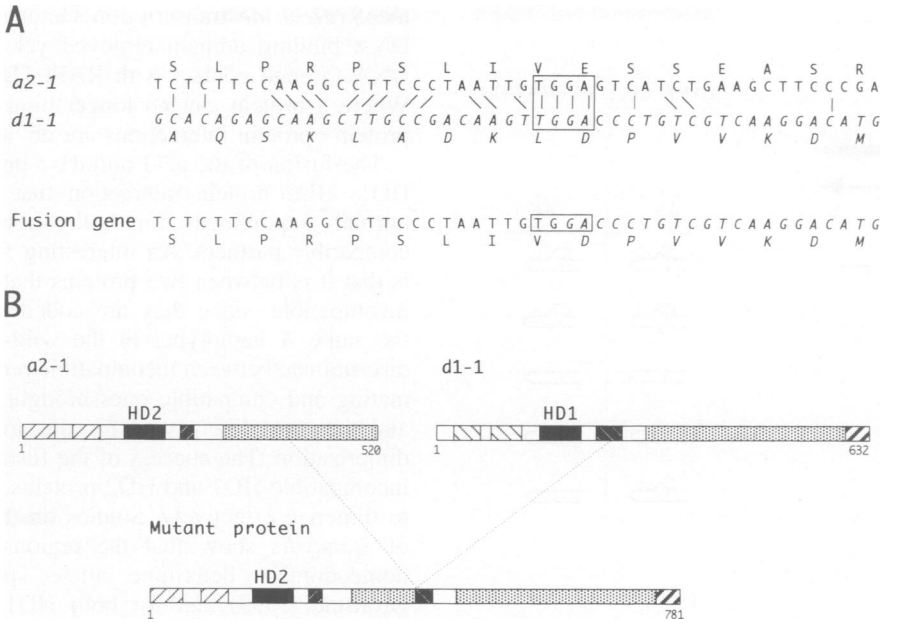

Fig. 5. (**A**) Partial DNA and predicted protein sequence of the *a2-1* and *d1-1* genes around the point of fusion in the mutant. DNA and protein sequences of the *A6* mutant shown below demonstrate the in-frame fusion of the *a2-1* and *d1-1* genes. The fusion point lies within the boxed 4 bp sequence. Other short homologies are indicated by lines. Note the *Hin*dIII restriction sites (AAGCTT) upstream of the fusion point in *d1-1* and downstream in *a2-1*. (**B**) Schematic representation of the a2-1 and d1-1 proteins and the mutant fusion protein. Predicted helical regions are shown as different striped boxes, homeodomains as black boxes and regions rich in proline, serine and threonine as stippled boxes.

selection system could detect them. The selection system used by Day (1963) was based on the need to have different *A* and *B* mating type genes to generate a fertile dikaryon that could produce fruit bodies. Partially compatible matings were made between monokaryons having different *B*s but the same *A*s and, as expected, fertile fruit bodies could only arise on these 'common *A*' heterokaryons if mutation occurred to generate a compatible *A* mating type. The mutations never generated new *A* specificities, but, like the mutant described in this study, conferred self-compatibility for *A* together with constitutive clamp cell development. The molecular analysis we describe here suggests why self-compatible mutations were so difficult to generate. The mutation involved an illegitimate recombination event which caused an in-frame fusion between an HD2 and an HD1 gene. This event was only likely to occur if the genes involved are transcribed in the same direction and in *A6*, these are *a2-1* and *d1-1* (as in the mutant described) or *b2-3* and *d1-1* (Figure 2). Day (1963) described other *A6* mutations (unfortunately no longer available) that mapped to the β complex and probably involved *b2-3 and d1-1*. Another critical factor in generating the mutation is the fusion point, which must bring together the essential functional domains of the two proteins. Preliminary analysis of another self-compatible mutation in *C.cinereus*, which arose in the *A43* haplotype, revealed that this also involved a major deletion (May *et al.*, 1991). We have recently re-analysed the wild-type *A43* complex and identified all the specificity genes (Kües *et al.*, 1994). From the deletion–fusion points mapped by May *et al.* (1991) we can predict that the deletion has fused an HD2 gene (*a2-2*) with an HD1 gene (*d1-1*) to generate a fusion product very similar to the one we describe here.

The *A6* fusion gene is sufficient to promote *A*-regulated development in the absence of any other specificity gene, because the deletion that generated it has eliminated the other active HD1 and HD2 genes from the *A6* locus. A wild-type *A6* phenotype cannot be restored by replacing these genes, as shown by introducing the mutant gene into an *A6* host and seeing that it still promotes clamp cell development (Figure 6). This is the basis of the dominant self-compatible phenotype; the mutant protein no longer needs to interact with another A protein in order to promote development. It may be noted that the clamp cell phenotype can only arise because the *A* genes are constitutively expressed in unmated as well as mated cells (Richardson *et al.*, 1993).

The fact that the mutant gene is derived by fusion of an HD2 and an HD1 gene is significant in terms of a compatible *A* mating. Genes encoding two classes of proteins with highly conserved HD1 and HD2 homeodomain motifs have been described in the mating type loci of four species of basidiomycete fungi; in the *A* loci of the mushrooms *C.cinereus* and *S.commune* and in the *b* loci of the smut fungi *Ustilago maydis* and *U.hordei*. Transformation studies in all species indicate that the compatible interaction is between an HD1 and an HD2 protein brought together by mating (Gillissen *et al.*, 1992; Specht *et al.*, 1992; Bakkeren and Kronstad, 1993; Kües *et al.*, 1994). An interaction between two dissimilar homeodomain proteins also occurs when cells of the yeast *Saccharomyces cerevisiae* mate. In this case the proteins encoded by the **a**1 and α2 mating type genes have been shown to dimerize to give a new transcription factor complex that binds specific DNA targets found upstream of developmentally regulated genes (Dranginis, 1990; Dolan and Fields, 1991; Mak and Johnson, 1993; Goutte and Johnson, 1994). Our molecular analysis of the *A6* mutant provides the first direct evidence that it is the

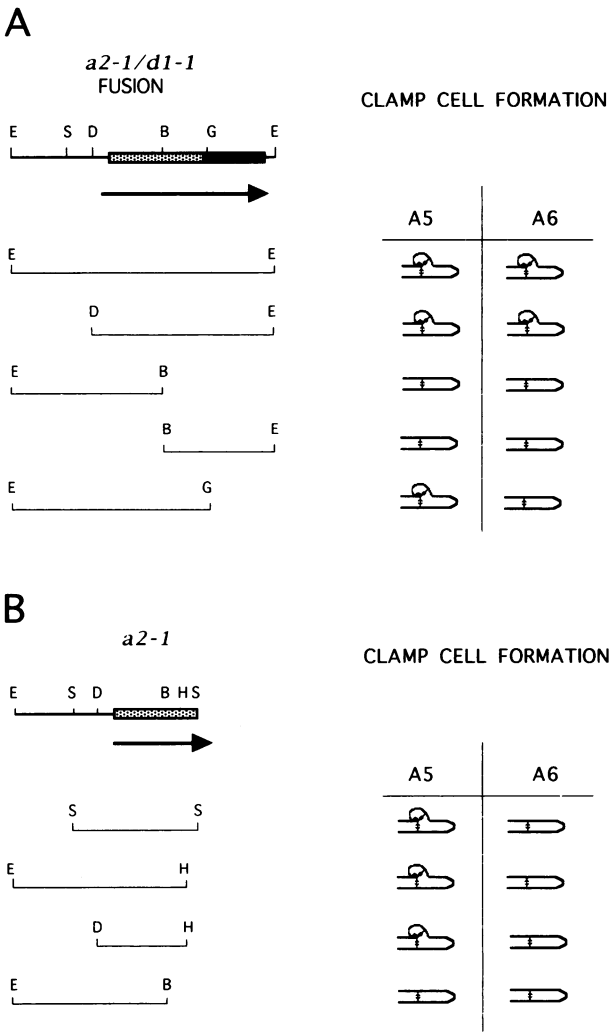

Fig. 6. (**A**) The ability of various complete or truncated forms of the *a2-1/d1-1* fusion gene and (**B**) the *a2-1* wild-type gene to induce clamp cell development when introduced into *A5* and *A6* host strains. The position of the coding sequence within the initial fragment is indicated by the bold lines. Restriction sites are shown as: B, *Bam*HI; D, *Dra*I; E, *Eco*RI; G, *Bgl*II; S, *Sal*I.

physical interaction between two dissimilar homeodomain proteins that triggers sexual development in basidiomycetes.

The predicted protein generated by the *A6* gene fusion contains almost all of the HD2 protein fused to the C-terminal half of the HD1 protein. The C-terminal region of the HD1 protein is essential for its constitutive activity, as shown by our gene truncation experiments, but the entire N-terminal region of the HD1 protein, including the HD1 homeodomain, is missing.

Assuming the fusion protein binds DNA, the HD2 homeodomain must be sufficient for correct target site selection. By analogy with the *S.cerevisiae* **a**1−α2 dimer one could expect that, in a normal interaction, both proteins bind DNA via their homeodomains. The forced dimerization may overcome the need for both proteins to bind the target sequence. Experiments with the fushi tarazu protein of *Drosophila* show that it can function normally without its homeodomain, suggesting that it is sufficient for it to form a complex with other DNA binding proteins (Fitzpatrick *et al.*, 1992; Ananthan *et al.*, 1993). Similarly, the *S.cerevisiae* transcription factor GCR1 can have its DNA binding domain removed yet still be attracted to DNA through contact with RAPI (Tornow *et al.*, 1993). Where a protein can no longer bind DNA, domains for protein−protein interactions are obviously crucial.

The fusion of the a2-1 and d1-1 proteins has forced an HD1−HD2 protein interaction that has overridden the normal requirement for both these proteins to find compatible partners. An interesting feature of the fusion is that it is between two proteins that would normally be incompatible, since they are coded by genes present in the same *A* haplotype. In the wild-type, the cell must discriminate between incompatible proteins present before mating and compatible ones brought together by mating and the most likely way for this to be achieved is by dimerization. The success of the fusion gene implies that incompatible HD1 and HD2 proteins are normally unable to dimerize effectively. Studies on the HD1 *bE* proteins of *U.maydis* show that the regions N-terminal to the homeodomain determine allele specificity (Yee and Kronstad, 1993) and for both HD1 and HD2 proteins these are thought to be likely dimerization interfaces (Gillissen *et al.*, 1992). It is significant that the N-terminal region of the d1-1 protein has been deleted in the A6 fusion protein, because it is no longer required for protein−protein recognition.

Sequence analysis of HD1 and HD2 genes (Tymon *et al.*, 1992; Kües *et al.*, unpublished observations) allows us to predict certain structural features of the proteins, as illustrated in Figure 5. Of particular relevance is the C-terminal region of the HD1 protein, which is clearly essential for promoting *A*-regulated development by the fusion protein. If it is removed, only a normal *a2-1* protein function is left. This region is rich in hydroxylated amino acids, proline and acidic residues and offers a potential activation domain (Tymon *et al.*, 1992). The fusion protein perhaps offers us an insight into why dimerization is required to give a functional transcription factor and what essential functions each of the two proteins contributes; the HD2 protein could be the main determinant of DNA binding specificity, whereas the HD1 protein could present an essential activation domain.

The fusion of two genes encoding putative transcription factors has led to the inappropriate expression of a developmental programme in *C.cinereus*. There are important parallels in mammalian cells where gene fusions disrupt normal development. Several cases of leukaemia can be attributed to chimeric transcription factors (Cleary, 1991; von Lindern *et al.*, 1992; Nucifora *et al.*, 1993) and, interestingly, some of these involve homeodomain proteins like the *A* proteins of *C.cinereus* (Kamps *et al.*, 1990, 1991; Nourse *et al.*, 1990; Dedera *et al.*, 1993). These chimeric mammalian proteins are generated by fusions between functionally unrelated transcription factors. The novelty in the protein we describe is that it is a fused dimer between two classes of proteins that normally have to recognize each other.

Materials and methods

Fungal strains, growth conditions and transformations

The wild-type *A6* strain was H9 (*A6B6*). The mutant *A6* strain, E117,9, is an adenine auxotroph (*A6mB5ade-8*) isolated by P.R.Day in 1967

from a fertile fruit body produced on a 'common *A*' heterokaryon following treatment with nitrosoguanidine (personal communication). Transformation hosts were the tryptophan auxotrophs LT2 (*A6B6 trp-1.1,1.6*) and FA2222 (*A5B5 trp-1.1,1.6*). General techniques for *C.cinereus* have been described by Lewis (1961). The minimal liquid and solid media were as described by Sealy-Lewis and Casselton (1978), with the addition of 100 mg/l adenine sulfate and L-tryptophan as appropriate. The complete medium was yeast malt glucose (YMG) (Rao and Niederpruem, 1969) supplemented with 100 mg/l L-tryptophan for growth of auxotrophs. Transformations were carried out by the protoplast procedure described by Casselton and de la Fuente Herce (1989). Plasmid DNA (1–2 μg) containing the *A* mating type genes was co-transformed with 1 μg pCc1001 containing the *C.cinereus trp-1* gene (Binninger *et al.*, 1987). TRP^+ transformants were selected and examined for clamp cell development. For nucleic acid extraction, small cultures initiated in liquid YMG were macerated and inoculated into liquid-supplemented minimal medium (for RNA) or YMG (for DNA) and grown on a rotary shaker for 48 h.

Plasmids and DNA methods

A6 mutant clones. Genomic DNA from the mutant *A6 C.cinereus* strain E117,9 was partially digested with *Mbo*I and fragments of 35–40 kb were ligated into the *Bgl*II cloning site of the cosmid vector LLC5200 constructed by Le Chevanton (Pukkila and Casselton, 1991). A single clone, p43B11, containing the mutant *A6* locus was isolated by screening with pCE1 and pCE12 (Mutasa *et al.*, 1990), which together contain the entire *A42* mating type locus and homologous flanking sequences (Kües *et al.*, 1992). A 4.5 kb *Eco*RI fragment derived from p43B11 and containing the mutant *A6* locus was subcloned into pBluescript KS− to give pWRF1.

A6 wild-type clones. A plasmid genomic library derived from the wild-type *A6* strain H9 cloned into the yeast vector YRp12 was kindly provided by Dr P.Pukkila (Pukkila *et al.*, 1984). This was screened with the 4.5 kb *Eco*RI fragment from pWRF1 and yielded pLAC1, containing *a2-1* and α-*fg*, and pLAC3, containing *d1-1* and β-*fg*. An *Eco*RI−*Hin*dIII fragment from pLAC1 containing a 3′ truncated *a2-1* gene was subcloned into pBluescript KS− to give pUA64. No clones containing the intervening region of the *A6* locus were detected in this plasmid library and a new library was constructed from H9 DNA partially digested with *Sau*3A and ligated into the *Bam*HI cloning site of the lambda vector GEM11 (Stratagene). The *b1-3* and *b2-3* genes were recovered on a 7.5 kb *Hin*dIII fragment and subcloned into pBluescript KS− to give pA62. pA626 contains a 2.2 kb *Xho*I fragment with the *b1-3* gene and pA625 a 2.6 kb *Xho*I−*Hin*dIII fragment with the *b2-3* gene. pUK2, containing the *a2-1* gene on a 2.1 kb *Sal*I fragment, and pESM2, containing the *d1-1* gene on a 4.0 kb *Bam*HI fragment, have been described by Kües *et al.* (1992) and are derived from *A42*. The *A42 d1-1* gene was chosen for the Northern analyses shown in Figure 4 to take advantage of a *Hin*dIII site not present in the *A6* allele. pAMT6 contains a 4.0 kb fragment from the non-coding region between the α and β loci (Kües *et al.*, 1992). mRNA isolation, Northern and Southern analyses and DNA sequencing were carried out according to Kües *et al.* (1992) and Tymon *et al.* (1992). High molecular weight *C.cinereus* genomic DNA for library construction and Southern analyses was isolated by the method of Mutasa *et al.* (1990). All other DNA methods were as described in Sambrook *et al.* (1989).

Acknowledgements

We thank Drs Flora Banuett, Alison Banham and Jane Mellor for helpful discussions. This work was supported by an AFRC postdoctoral fellowship, AFRC grant PG43/548 and SERC grant GR/F78781 to L.A.C. B.G. was supported by an SERC studentship and by the Gatsby Charitable Foundation. W.V.J.R. was supported by a Sainsbury studentship from the Gatsby Charitable Foundation.

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Received on May 12, 1994; revised on June 14, 1994