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Wild-type and mutant alleles of the *Aspergillus nidulans* developmental regulator gene *brlA*: correlation of variant sites with protein function

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Abstract The DNA sequences of two wild-type and eleven mutant alleles of the developmental regulator gene *brlA* from *Aspergillus nidulans*, which encodes a zinc-finger protein, were characterized. Variant sites were located on rescued plasmids or PCR products based either on their meiotic map position or the use of denaturing gradient gel electrophoresis. Mutations in three null mutants, one of which is partially suppressible, encode premature stop codons. Two environmentally sensitive mutants were characterised by substitution of leucines required for stabilisation of α-helices in each of the two putative zinc-finger domains. A third zinc-finger substitution is predicted to disrupt recognition of a guanine residue in the DNA target. The mutations in four other leaky mutants map C-terminal to the zinc fingers; one minimally leaky mutant has a premature stop codon, which results in the removal of the last 38 residues of the protein product.

Key words *brlA* mutants · Denaturing gradient gel electrophoresis (DGGE) · Suppressible mutant · Mutant site detection · Zinc finger protein

Introduction

Molecular analysis of conidial development in *Aspergillus nidulans* (reviewed by Timberlake 1991; Timberlake and Clutterbuck 1994; Adams et al. 1998) was initiated by the characterisation of mutants at a number of loci including *bristle* (*brlA*), which encodes a regulatory zinc-finger protein (Clutterbuck 1969; Adams et al. 1988; Chang and Timberlake 1993). The conidiophores of null *brlA* mutants consist of an undifferentiated bristle-like stalk, whereas those of morphologically leaky *brlA* mutants show a variety of phenotypes, ranging from a bristle with only a rudimentary vesicle-like expansion bearing a few long branches to conidiophores with near-normal vesicles surmounted by numerous multi-tiered, metula-like cells (Clutterbuck 1969). The bristles of null mutants are unpigmented, unlike the structures in the wild type and in all morphologically leaky mutants, which have a grey-brown pigmentation, due to activation of the genes *ivoA* and *ivoB* (Birse and Clutterbuck 1990, 1991; Clutterbuck 1990). Although the conidiophores of *brlA14* and *brlA19* strains are unpigmented and morphologically undifferentiated, they express appreciable levels of the *ivoB*-encoded enzyme *N*-acetyl-6-hydroxytryptophan oxidase (AHTase) (see Table 1). Thus, expression of the *ivoB* locus appears to be a very sensitive indicator of $BrlA^+$ activity.

The *brlA* gene has been cloned (Johnstone et al. 1985; Boylan et al. 1987) and sequenced (Adams et al. 1988). Deletion of either of its two zinc-finger motifs, or substitution of the essential cysteine residues in these motifs, results in a null phenotype (Adams et al. 1988, 1990). Regulation of *brlA* is complex; there are two major transcripts, which are believed to be regulated by a combination of translational control by an upstream mini-ORF, developmental regulation of unknown nature, and positive transcriptional feedback via the *abaA* gene product (Prade and Timberlake 1993; Han et al. 1993). Proteins encoded by the two transcripts differ by 23 N-terminal residues but the two polypeptides are functionally at least partially interchangeable (Prade and Timberlake 1993), and all mutations discussed here lie within the common sequence.

The alleles *brlA12*, which shows position-effect variegation (Clutterbuck 1970; Clutterbuck and Spathas

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1984), and *brlA17*, which has a null but partially suppressible phenotype (Clutterbuck et al. 1992), both map in the 5′ half of the gene. However, the meiotic map of *brlA* (Clutterbuck et al. 1992) suggests that null mutations are scattered through the gene but that the majority of leaky mutations lie in the 3′ half, possibly in the region that codes for the presumptive DNA-binding zinc-finger motifs. In the work reported here, we confirm the postulated concentration of non-null (leaky) mutations in the zinc-finger and C-terminal regions, and show that three of these mutations have readily interpretable functional effects on the zinc-finger motifs, while a fourth leads to truncation of the polypeptide by 38 residues.

Materials and methods

Genetic techniques

Aspergillus methods were as described (Pontecorvo et al. 1953; Clutterbuck 1974) and strains were derived from the Glasgow collection (http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/). All *brlA* mutants were derivatives of the *biA1*; *veA1* strain (G051), which in turn is a derivative of the wild type G00 (FGSC4). The wild isolates of *A. nidulans* −33, 66, 89 and 94 (Glasgow strain numbers G0033 and 0033-1, G0066, G0089-2 and G0094.2, respectively) – were originally obtained from the Birmingham collection (Grindle 1963; Butcher 1968). Other *A. nidulans* strains used were: AJC528a19 (*yA2*; *argB2*; *pyroA4*; *veA1 niiA4 brlA17 cnxA5 ivoB63*), SDM380 (*pabaA1*; *alX4*; *sB43*; *suaC109*; *fwA1*; *veA1* obtained from Dr. S.D. Martinelli), and G34 (*yA2*; *methH2 argB2*; *veA1*). Mycelia for DNA extraction were grown at 37° C for 30 h as surface cultures (Birse and Clutterbuck 1990).

Mutants were tested for phenotypic suppression by the aminoglycoside antibiotic paromomycin (Martinelli and Roberts 1983), by inoculating them on agar plates of complete medium in a pattern of streaks radiating from a well to which solid paromomycin was added after 24 h. The AHTase activity of such colonies was tested by overlaying the plates with filter paper soaked in 67.2 mM hydroquinone monomethyl ether in 0.5 M ammonium tartrate (pH 7.0), activity being detected as brown staining (Birse and Clutterbuck 1990).

DNA manipulations

Genomic DNA was prepared according to standard procedures (Raeder and Broda 1985; DuTeau and Leslie 1991). Appropriate oligonucleotide primers (see below) were designed according to the published *brlA* sequence (Genbank Accession No. M20631) to amplify a 1415-bp fragment which included the whole coding region of the *brlA* α-transcript. Nucleotides are numbered from the α-transcription start site (Adams et al. 1988).

pBRL17 was obtained by the steps shown in Fig. 1; an *argB2*; *brlA17* strain (AJC528a19) was transformed by homologous chromosomal integration with plasmid pILJ421, which carries the *argB*$^+$ selective marker and the *brlA42* mutant allele. Approximately 15% of transformant colonies had the Arg$^+$ Brl$^+$ phenotype and could have arisen either by reciprocal recombination, giving one wild-type and one double mutant allele or, as illustrated in Fig. 1, by crossing over accompanied by gene conversion (a frequent occurrence; A. J. Clutterbuck, unpublished), which leaves the second allele as *brlA17*. All wild-type transformants formed occasional sectors, some of which were Arg$^+$ and had the null phenotype conferred by *brlA17*; these are assumed to have arisen by vegetative gene conversion between tandem *brlA* genes: i.e. from *brlA*$^+$-*brlA17* to *brlA17*-*brlA17*. DNA prepared from one such

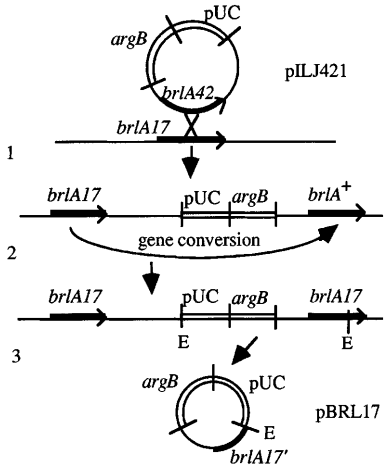

Fig. 1 Origin of pBRL17. The *brlA* gene is shown as a *heavy line*; the *arrow* indicates the direction of transcription. Flanking chromosomal DNA is shown as a *thin line*, and the *argB* gene and pUC vector sequences are represented by *open boxes*. Step 1: transformation of an *argB2*; *brlA17* strain with pILJ42, with homologous integration in the *brlA* gene to give a *brlA*$^+$ recombinant. Step 2: vegetative gene conversion to give duplicate copies of the *brlA17* allele. Step 3: restriction with *Eco*RI (E, *Eco*RI site), ligation and rescue of pBRL17 into *E. coli*. See text for further details

purified sector was digested with *Eco*RI, diluted, re-ligated and used to transform *E. coli* strain DH5 [F$^-$, *recA*1, *endA*1, *gyrA*96, *thi*–1, *hsdR*17, {r$^-$,m$^+$}, *supE*44]. The pBRL17 plasmid thus recovered contained the 3′ portion of the *brlA17* allele, up to an *Eco*RI site at nucleotide 1541. We did not determine whether it also carried the *brlA42* mutation.

PCR amplifications were performed in 25-μl volumes containing Promega 1× *Taq* polymerase buffer, 0.33 μM of each primer, 0.2 mM of each dNTP, 1 U of *Taq* DNA polymerase (Promega; M166) and 25–50 ng of genomic DNA. Amplifications were conducted in a Hybaid Thermal Reactor programmed for one cycle of 3 min at 94° C, 35 cycles of 1 min at 94° C, 1 min at 60° C and 2 min at 72° C and one cycle of 5 min at 60° C, 10 min at 72° C.

A 2-μl aliquot of the PCR reaction product was analysed by electrophoresis in 1.2% agarose after staining with ethidium bromide. A 7-μl sample of the remainder was digested with either *Rsa*I, *Hae*III or *Sau*3A1 and *Kpn*I in a 25-μl volume. Denaturing gradient gel electrophoresis was conducted in a Hoeffer DGGE apparatus according to the method of Myers et al. (1987). Denaturing gradients (40–80% or 30–80% urea/formamide; 100% is equivalent to 40% formamide, 7 M urea) were produced in 6.5% polyacrylamide gels (37.5:1 acrylamide/bisacrylamide). Electrophoresis was conducted for 20 h at 80 V/200 mA. Banding patterns were visualised by staining with ethidium bromide. Since the mobility of fragments in DGGE gels is not always proportional to fragment size, bands were excised from the DGGE gel and electrophoresed on a 1.2% agarose gel to confirm their identity.

For sequencing, a separate amplification from genomic DNA was conducted using the appropriate primers, and 20-μl aliquots were electrophoresed on a 1.2% agarose in TBE buffer. The PCR product band was excised from the gel, purified by centrifugation through glass wool (6000 ×*g*, 1 min) and precipitated with ethanol. Single-stranded DNA was prepared from both strands of the purified PCR product by asymmetric PCR with a single primer in a 40-μl reaction. Otherwise amplification conditions were as above. After precipitation with 0.65 volumes of isopropanol and 0.1 volume of sodium acetate (pH 5.2), sequencing was carried out using Sequenase dideoxy-sequencing reagents (USB) and electrophoresis on standard sequencing equipment (Sequigel II, Bio-Rad).

The following primers were used for PCR and sequencing: P838 (5′-CGTAAACTGAACGGTGCTCTGG-3′; 22 nt, 268–289), P1041 (5′-TTCGATGCGCAGTGC-3′; 15 nt; 570–584), P2184 (5′-

AACTCGGGACTCGCTCG-3′;17 nt; 910–894), P903 (5′-GACGACAGACCTCTAAGC-3′; 18 nt; 1241–1258), P993 (5′-TTGAGTGGCTCTTCATG-3′;17 nt; 1384–1368), P966 (5′-CCAGGCTCATATCGC-3′; 15 nt; 1630–1616), and P839 (5′-AGATTCCATGCAGATCAGCCCTC-3′; 23 nt; 1682–1660).

Results and discussion

DNA sequences of *brlA* alleles

Alleles selected for sequencing (Table 1) included two phenotypically null mutations (*brlA4* and *-23*), the partially suppressible null *brlA17*, and eight that were in some sense leaky (*brlA6*, *-7*, *-9*, *-10*, *-14*, *-19*, *-35* and *-42*). All behaved as point mutations in crosses (Clutterbuck et al. 1992). The molecular basis of the variegated mutant *brlA12*, which carries a III–VIII translocation whose breakpoint on chromosome VIII is close to the *brlA* locus (Clutterbuck 1970), will be described elsewhere.

Sites of mutation in *brlA42* and *brlA17* were determined by sequencing appropriate segments, as predicted by the meiotic map (Clutterbuck et al. 1992), of plasmids pILJ421 (Johnstone et al. 1985) and pBRL17, respectively. The sites of the mutations in *brlA4*, *-6*, *-7*, *-9*, *-10*, *-14*, *-19*, and *-35* were deduced from the recombination map to lie in the 3′ region of the gene, which was therefore amplified from genomic DNA of appropriate mutant strains using the primers indicated in Table 1, and sequenced with the same primers. For wild types and mutations whose positions were less certain, a full length (1415 bp) PCR product, obtained using primers P838 and P839, was fragmented with various restriction enzymes. Restriction fragments predicted by the TRAVEL programme (Lerman and Silverstein 1987) as likely to show single base changes, were then subjected to DGGE. Regions identified by this means as showing mutations were then sequenced from the large PCR product using primers indicated in Table 1, which lists the base substitutions found and the predicted amino acid changes. Comparison of DGGE band shifts with sequence changes (Table 1) showed that A/T to G/C base changes (e.g. WT0066: A to G) or vice-versa (e.g. WT0094.2: C to T) were visualised, respectively, as an increase or decrease in band mobility. Figure 2 shows the mutation sites in relation to the published wild-type peptide sequence.

Altogether, the two wild-type and eleven mutant alleles contained eleven transitions and three transversions. One allele, *brlA14*, had two base substitutions in the same codon, but the remainder all showed single base substitutions. Wild type 94 differed from the Glasgow strain *brlA* DNA sequence by a silent base substitution; the other wild type studied (66) had a non-conservative substitution in a poorly conserved region (Table 1, Fig. 2).

Correlation of mutations with BrlA protein function

Crystallographic analysis of zinc finger protein-DNA complexes (Elrod-Erickson et al. 1996), experimentally

Table 1 Base and amino acid changes in *brlA* mutant and wild-type variants

Allele	Mutagen[a]	Primers	Base change[b]	Amino acid change[c]	Equivalent residue in other *Aspergillus* species[d]			Morphological phenotype[e]	AHTase[f]
					A. fumigatus	*A. oryzae*	*A. terreus*		
w.t. 94	–	1041, 2184	C669T	None				Wild-type	n.t.
brlA17	UV	1041, 2184	G700T	E118*				Suppressible null	5.0
w.t.66	–	1041, 2184	A752G	Q135R	H	H	P	Wild-type	n.t.
brlA23	NTG	903, 993	C1297T	Q317*				Null	0
brlA4	DES	903, 966	C1348T	Q334*				Null	0
brlA7	UV	903, 966	T1358C	L337S	L	L		Leaky (c)	199.5
brlA10	NTG	903, 966	G1364A	R339K	R	R	R	Leaky (b)	69.7
brlA42	NA	903, 966	C1447T	L367F	L	L	L	Leaky (c), temperature sensitive	100
brlA14	UV	903, 966	T1498G C1500T	Y384D	Y	Y		Null	53.2
brlA35	DES	903, 966, 839	T1502C	V385A	V	V		Leaky (b-c)	162.5
brlA19	UV	903, 966	C1533A	Y395*				Null	83.7
brlA6	NA	903, 966	G1568A	G407E	G	G		Leaky (a-b)	98.9
brlA9	NTG	903, 966	C1573T	P409S	P	P		Leaky (b)	137.1

[a] Mutagens: UV, ultraviolet light; NTG, N-methyl-N′-nitro-N-nitrosoguanidine; DES, diethyl sulphate; NA, nitrous acid

[b] Nucleotide sequence numbered as in Han et al. (1993) from the α-transcript start site

[c] Polypeptide sequence numbered from the start of the α-polypeptide. Asterisks indicate the presence of a premature termination codon at the site indicated

[d] For amino acid substitution mutations, the degree of conservation of the residue is indicated by comparison with the aligned *brlA* sequence from *A. oryzae* (Y. Osanu, L. Byung Rho and G. Katuya, unpublished; Genbank Accession No. D89010), *A. fumigatus* (T.H. Adams, personal communication), and where available, *A. terreus* (Hore 1998)

[e] Letters in *parentheses* refer to Fig. 2 in Clutterbuck (1990) in which (a) represents the *brlA* null phenotype, (b) is intermediate and (c) represents the most leaky phenotype that is still devoid of conidia

[f] N-acetyl-6-hydroxytryptophan oxidase activity of surface cultures, derived from Clutterbuck (1990). Activities are expressed as a percentage of that for *brlA42* after subtraction of an *ivoB*⁻ blank. n.t. = not tested

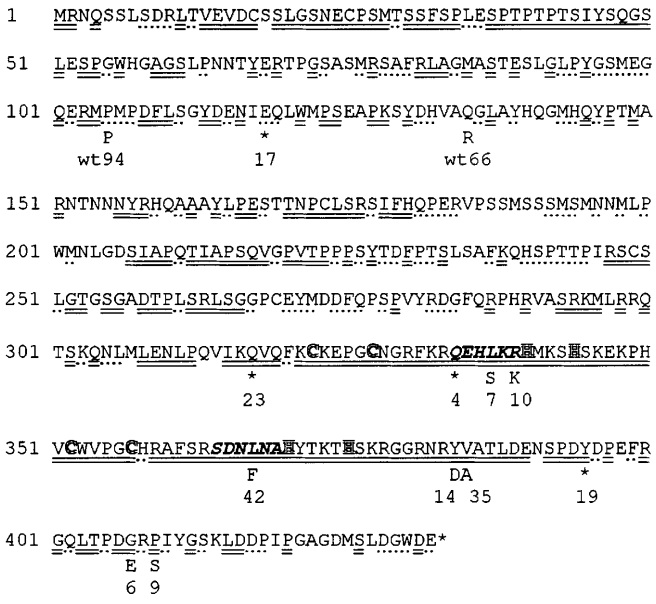

Fig. 2 The sequence of the α-polypeptide encoded by *brlA* (from Adams et al. 1988, Genbank Accession No. M20631). Mutant and wild-type variants are shown *below* the Glasgow wild-type sequence; the numbers refer to the mutant alleles and wild type (wt) strain numbers . Residues conserved in either *A. oryzae* (Y. Osanu, L. Byung Rho and G. Katuya, unpublished; Genbank Accession No. D89010) or *A. fumigatus* (T. H. Adams, personal communication) are shown by *dotted underlining*, those conserved in both species are marked by *double underlining*. The zinc-coordinating cysteine and histidine residues of the two postulated zinc fingers (Adams et al. 1988) are printed in *outline script*, and the α-helix/DNA contact region is printed in *bold italics*

designed interacting sites (Desjarlais and Berg 1994; Choo and Klug 1997; Greisman and Pabo 1997) and molecular modelling of wild-type and mutant DNA-protein interactions (Espeso et al. 1997) make it possible to predict the effects on DNA binding of the three mutations (*brlA7*, *-10* and *-42*) that have amino acid substitutions in the zinc-finger motifs. In *brlA7*, a leucine residue required for stability of the first zinc-finger α-helix, which makes contact with the target DNA, is replaced with a serine residue. This hydrophobic to polar substitution is associated with a moderately severe bristle phenotype, which can be partially suppressed by high osmolarity (Clutterbuck 1969). In *brlA42*, the corresponding leucine in the second finger is replaced, more conservatively, by phenylalanine. The instability caused by this substitution gives a predictably less severe phenotypic defect that is cured by growth at lower temperatures. In *brlA10* a lysine residue replaces an arginine which is predicted to bind to the first guanine in the "bristle response element" (MRAGGGR), as deduced by Chang and Timberlake (1993). The resulting phenotype is more severe than that of either *brlA7* or *brlA42* and is not alleviated by altering environmental conditions (Clutterbuck 1969).

The four developmentally null mutants analysed all carry premature stop codons. *brlA23* is a mutation to UAG (amber), while *brlA17*, *-4* and *-19*, are mutations to UAA (ochre). Of these, *brlA19*, which has a stop codon 38 residues from the C-terminal, has appreciable AHTase activity although it is unpigmented and morphologically null. This implies that the missing C terminal is partially dispensable for activation of *ivoB*, although apparently required for activation of *ivoA* (because the mutant lacks pigment; Clutterbuck 1990) and of genes determining morphological development. A second mutant with the same phenotype, *brlA14*, has a tyrosine to aspartic acid substitution in the same region.

Phenotypic suppression of *brlA17*

As briefly reported earlier (Clutterbuck et al. 1992), all *brlA* mutants were tested for translational suppression by paromomycin (Roberts et al. 1979; Martinelli and Roberts 1983). The only *brlA* mutants that showed any morphological amelioration of the phenotype were *brlA12* and *brlA17*. Suppression of *brlA17* was clear, but limited in extent, with minimal vesicle formation and bristle forking. It is doubtful whether the AHTase level in normal surface cultures of *brlA17* (see Table 1) is significantly above background, but AHTase activity was readily detected in this strain on the paromomycin test plate. When *brlA17* was crossed to strain SDM360, which carries the ribosomal supersuppressor *suaC109* (Roberts et al. 1979; Martinelli 1984), approximately 50% of the Brl^- progeny showed a partially suppressed phenotype, similar to that obtained with paromomycin.

Of the three ochre mutations, only *brlA17* is suppressible by *suaC109* and by paromomycin; it is therefore evident that suppression is dependent on factors other than the nature of the stop codon concerned. Furthermore, it is clear that suppression by *suaC* is not codon specific, since the only other *suaC109*-suppressible mutation of *A. nidulans* that has been sequenced (*areA600*) is an amber (UAG) mutation (Al Taho et al. 1984; Kudla et al. 1990).

The distribution of mutations within the gene and correlation with the genetic map

The amino acid substitutions in all eleven leaky mutants occur in the highly conserved zinc-finger region or the C-terminal tail. Failure to find amino acid substitution mutants in the N-terminal three-quarters of the gene suggests that most substitutions in this region are phenotypically silent. This explanation is supported by the relatively low degree of conservation of much of this sequence (Fig. 2), and the non-conservative, but phenotypically silent, substitution in the wild-type strain 66. It is therefore striking that the spacing between conserved regions at the 5′ end of the gene and zinc finger region at the 3′ end is similar in all three species sequenced. An alternative, but less likely explanation for the failure to find leaky mutations in the 5′ half of the gene is that substitutions here give lethal or unrecognized phenotypes, e.g. that due to *brlA* overexpression (Adams et al. 1988).

The intragenic meiotic map of the *brlA* gene (Clutterbuck et al. 1992) was obtained from crosses to three standard mutants, *brlA17*, *-39* and *-9*. Mutants were mapped by crossovers within the gene into six groups, ordered by reference to outside markers. This distribution is confirmed by the order of *brlA* mutants sequenced here: *brlA17* (group B), *brlA23* (group E), *brlA4*, *-7*, *-10* and *-42* (group F) and *brlA14*, *-35*, *-19*, *-6* and *-9* (group G). On the other hand, ordering mutants by recombination frequencies within groups was considered less reliable, and is indeed confirmed here as being ineffective. No recombinants were detected in crosses between *brlA9* and other mutants in group G, at the 3′ end of the gene. Since these mutations are now seen to be a maximum of 73 bp apart, failure to detect recombinants in platings of approximately 10^5 spores is unsurprising.

Comparison of recombination frequencies with DNA intervals (Clutterbuck et al. 1992), gives values between 6 kb and115 kb per map unit, with a median of 48 kb. The distance between *brlA* and *pyrD* is 4.3 map units (A. Y. Aleksenko, unpublished; see web site: http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/) corresponding to 20 kb (Kupfer et al. 1997), giving a ratio of 4.7 kb per map unit, which is close to the estimated average of 5.2 kb per map unit for the whole *A. nidulans* map (Clutterbuck 1992). Recombination within the *brlA* gene is therefore infrequent in comparison with intergenic recombination in this region. There is a slight, but not statistically significant, correlation between the position of the midpoint of the interval mapped and recombination frequency per nucleotide, showing a maximum at the 5′ end, in agreement with the previously observed gradient of recombination frequency (Clutterbuck et al. 1992).

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