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The regulatory gene *are*A mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc finger

Bernard Kudla[1,4,7], Mark X.Caddick[1,2,7], Tim Langdon[1], Nilce M.Martinez-Rossi[1,5], Christopher F.Bennett[1], Susan Sibley[3], R.Wayne Davies[3,6] and Herbert N.Arst, Jr[1,*]

[1]Department of Bacteriology, Royal Postgraduate Medical School, Ducane Road, London W12 0NN, [2]Department of Genetics and Microbiology, University of Liverpool,. PO Box 147, Liverpool L69 3BX, UK, and [3]Allelix Biopharmaceuticals, Inc., 6850 Goreway Drive, Mississauga, Ontario L4V 1P1, Canada

[4]Present address: Laboratoire de Biologie et Génétique Moléculaire, Bâtiment 400, Université Paris-Sud, 91405 Orsay Cedex, France

[5]Present address: Departmento de Genética, Facultade de Medicina de Ribeirão Preto-USP, 14.049 Ribeirão Preto, SP, Brazil

[6]Present address: Robertson Institute of Biotechnology, Deparment of Genetics, University of Glasgow, Church Street, Glasgow G11 5JS, UK

[7]The first two authors contributed equally to this work

*To whom correspondence should be addressed

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The regulatory gene *are*A mediating nitrogen metabolite repression in *Aspergillus nidulans* has been sequenced and its transcript mapped and orientated. A single ORF can encode a protein of 719 amino acids. A 52 amino acid region including a putative 'zinc finger' strongly resembles putative DNA binding regions of the major regulatory protein of erythroid cells. The derived protein sequence also contains a highly acidic region possibly involved in gene activation and 22 copies of the motif S(T)PXX, abundant in DNA binding proteins. Analysis of chromosomal rearrangements and transformation with deletion clones identified 342 N-terminal and 124 C-terminal residues as inessential and localized a C-terminal region required for nitrogen metabolite repressibility. A −1 frameshift eliminating the inessential 122 C-terminal amino acids is a surprising loss-of-function mutation. Extraordinary basicity of the replacement C terminus might explain its phenotype. Mutant sequencing also identified a polypeptide chain termination and several missense mutations, but most interesting are sequence changes associated with specificity mutations. A mutation elevating expression of some structural genes under *are*A control whilst reducing or not affecting expression of others is a leucine to valine change in the zinc finger loop. It reverts to a partly reciprocal phenotype by replacing the mutant valine by methionine.

Key words: *are*A/*Aspergillus nidulans*/erythroid transcription factor sequence homology/nitrogen metabolite repression/zinc finger

Introduction

The fungus *Aspergillus nidulans* has exceptional nutritional versatility, utilizing, for example, a wide range of nitrogen sources. Such metabolic versatility implies the possession of numerous enzymes and permeases needed only under certain growth conditions. The advantages of efficient regulation of gene expression in such an organism are apparent. Nitrogen metabolite repression of the syntheses of enzymes and permeases involved in nitrogen acquisition enables favoured nitrogen sources such as ammonium and L-glutamine to be utilized preferentially. Nitrogen metabolite repression in *A.nidulans* is mediated by the positive-acting regulatory gene *are*A whose protein product is thought to act at transcription (reviewed by Arst and Scazzocchio, 1985; Wiame *et al.*, 1985). Loss-of-function mutations, designated *are*A^r, result in an inability to utilize nitrogen sources other than ammonium and glutamine and low to undetectable levels of enzymes and permeases under *are*A control. Very rare mutations, designated *are*A^d, lead to derepressed expression of activities under *are*A control. In the wild type glutamine and ammonium (through conversion to glutamine) prevent the expression and/or activity of the *are*A product (reviewed by Wiame *et al.*, 1985).

The *are*A gene is a particularly attractive eukaryotic regulatory gene for study because of the large number of structural genes under its control and the ease with which expression of these can be monitored. These factors plus the apparent subtlety in the relationship of the *are*A protein to its receptor sites have enabled collection of a uniquely diverse set of mutant phenotypes (reviewed in Arst and Cove, 1973; Wiame *et al.*, 1985). Here we localize the transcribed region of *are*A, present and analyse the sequence of *are*A and its environs, use chromosomal rearrangements and transformation with deletion clones to identify non-essential regions of the protein and a region involved in the ability of glutamine (and ammonium) to exert nitrogen metabolite repression, and identify a number of mutant sequence changes. Of particular interest are a pair of mutations altering the specificity of structural gene activation by *are*A and having a reciprocal or 'mirror image' phenotype such that activities elevated by one are reduced by the other and *vice versa*. These mutations alter a central residue in the loop of a putative zinc finger, presumably identifying that residue as involved in the efficiency of recognition of at least some *are*A receptor sites. Relatively conservative amino acid changes in that position are responsible for considerable differences in phenotype.

Results and discussion

Subcloning areA

Caddick *et al.* (1986) isolated a λ clone with an 11.6 kb insert containing *are*A. We were able to complement the translocation-associated loss-of-function mutation *are*A^r-18 (Rand and Arst, 1977; Arst, 1981; Caddick *et al.*, 1986; Arst *et al.*, 1989b) with a 2.8 kb *Bam*HI−*Kpn*I clone pBK (Figure 1) and identify by Southern blotting *are*A$^+$ transformants containing a single copy of the clone integrated

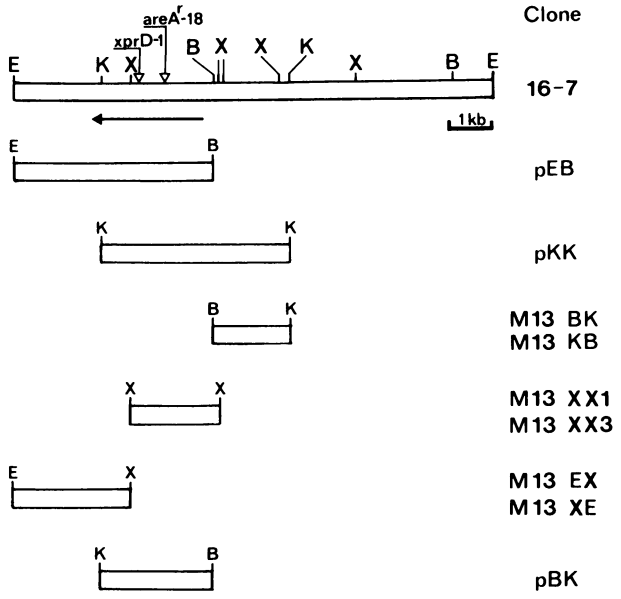


Fig. 1. Subcloning of *are*A from the original λ clone 16−7 (Caddick *et al.*, 1986). Restriction sites for *Bam*HI (B), *Eco*RI (E), *Kpn*I (K) and *Xho*I (X) and approximate positions of the *are*A^r-18 translocation and *xpr*D-1 inversion breakpoints are indicated. A horizontal arrow indicates the position and orientation of the *are*A transcript as determined by Northern blotting and nuclease protection experiments. Details of subcloning are given in Materials and methods.

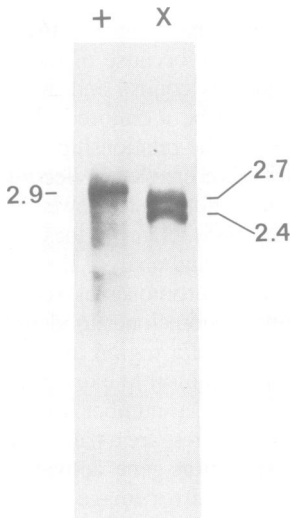


Fig. 2. Northern blot analysis of polyadenylated RNA from wild type (+) and *xpr*D-1 (X) strains. The strains were grown for 16 h at 37°C with 100 μg/ml uric acid as sole nitrogen source. 10 μg of RNA was electrophoresed in a 1.5% agarose gel and blotted. The blot was then hybridized with the labelled 2.1 kb *Xho*I fragment (equivalent to that cloned into M13 XX1 and M13 XX3—see Figure 1). Approximate sizes (in kb) of bands are shown at the sides.

heterologously (not shown). This 2.8 kb clone overlaps the *are*A^r-18 and *are*A^d-101 translocation breakpoints and the (allelic) *xpr*D-1 inversion breakpoint (Caddick *et al.*, 1986; Arst *et al.*, 1989b).

Identification and orientation of the areA transcript

In Northern blots *are*A-containing clones hybridize to a poly(A)$^+$ mRNA of ~2.9 kb in the wild type (Figure 2). In a strain carrying the *are*A-truncating inversion *xpr*D-1 (Arst, 1982; Caddick *et al.*, 1986; see below) two hybridizing poly(A)$^+$ mRNAs, 2.4 and 2.7 kb, are evident. When Northern blots of wild type were probed with single stranded M13 clones, the direction of transcription was found to be towards the centromere (i.e. right to left on the restriction map in Figure 1) (Data not shown).

DNA sequence analysis

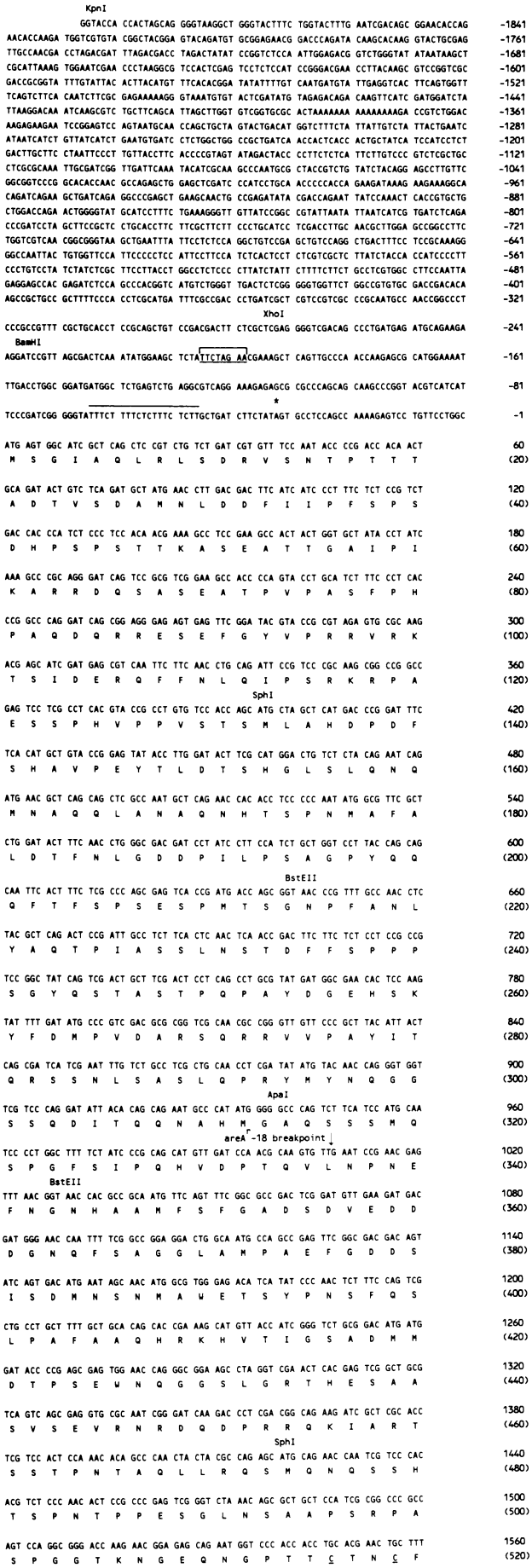
The sequence of a 4986 bp region containing the 2.8 kb *Bam*HI−*Kpn*I *are*A-complementing fragment is shown in Figure 3. Figures 4 and 5 show the results of nuclease protection experiments to map the 5′ and 3′ ends of the transcript. The transcribed region is ~2709 nucleotides long, which when polyadenylated agrees tolerably well with the 2.9 kb size estimated from Northern blots.

Two features of the DNA sequence immediately upstream of the transcribed region deserve attention. One is a pyrimidine-rich region between −65 and −47 (relative to the start of translation). This is a frequent feature of the immediate 5′ regions of many fungal structural genes (Ballance, 1986; Gurr *et al.*, 1987). The other is a copy of the 8 bp yeast heat shock element (Tuite *et al.*, 1988) ~200 bp upstream of the ORF. The significance of this sequence is unclear because preliminary experiments suggest that heat shock does not increase transcription of *are*A (M.X.Caddick, unpublished).

The 3′-untranslated portion of the transcript is rather long (~539 bp) and includes, in addition to a possible polyadenylation signal, one imperfect and three perfect contiguous copies of a 7 bp sequence directly repeated. The perfect copies each contain an *Acc*I site. No function of this repeated sequence is apparent, at least from *in vivo* studies, as truncation at the middle of the cluster of *Acc*I sites or 32 codons from the 3′ end of the ORF affects neither gene activation by the *are*A product nor nitrogen metabolite repressibility (see below).

Derived protein sequence

Within the transcribed region there is a single long open reading frame (ORF) of 2157 bp, reading towards the centromere. Several nuclease protection experiments (not shown) using fragments from within the transcribed region have given no evidence for the existence of introns nor would any necessarily be expected from the relationship between the size of the transcribed region and the apparent mRNA size. A computer search failed to reveal the existence of any putative intron splice or lariat sites. The derived 719 amino acid sequence gives a protein with a calculated molecular mass of 76 708 daltons and several notable features. Firstly beginning at residue 516 there is a putative zinc finger DNA binding motif (Klug and Rhodes, 1987) of structure Cys-X_2-Cys-X_{17}-Cys-X_2-Cys. Secondly the sequence is rich in the S(T)PXX motif identified by Suzuki (1989) as occurring frequently in DNA binding proteins. It contains 13 SPXX and nine TPXX motifs, corresponding to frequencies (Suzuki, 1989) of 18.2×10^{-3} and 12.5×10^{-3}, respectively, placing it towards the top of the range for DNA binding proteins. Thirdly, a charge distribution profile (Figure 6) indicates a highly acidic region between residues 330 and 400. Acidic regions, particularly if they form amphipathic α-helices, have been implicated in transcriptional activation by regulatory proteins (Giniger and Ptashne,



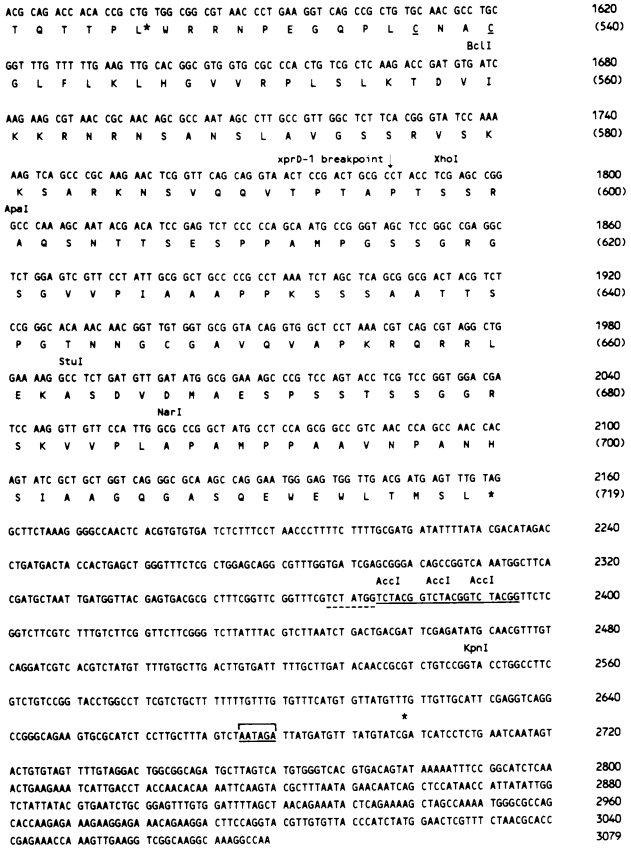


Fig. 3. DNA sequence of a 4986 bp genomic sequence including *are*A. The derived protein sequence of the 2157 bp ORF is shown in single letter codes and numbered in brackets. Cysteine residues forming the putative zinc finger are underlined and the leucine residue replaced by the *are*A-30, -31 and -102 mutations is starred. Numbering of nucleotides begins with the initiation codon. The 5′ (at −33) and 3′ (at +2699) limits of transcription are starred. Restriction sites important to this work are shown as are the *are*A^{r}-18 translocation and *xpr*D-1 inversion breakpoints. The 8 bp yeast heat shock consensus sequence (beginning at −206) and possible polyadenylation signal (beginning at +2675) are boxed. The 5′ pyrimidine-rich sequence (beginning at −65) is overlined and the 7 bp contiguous direct repeats in the 3′ untranslated region (beginning at +2368) are underlined, solidly for perfect copies and interruptedly for the imperfect copy. The sequences CAAAT and TATA occur, beginning at −223 and −36, respectively, but it is unclear whether either is important.

1987; Hope *et al.*, 1988). Of particular note are sequences beginning at residue 354 where six of eight residues are Asp or Glu and at 375 where these amino acids account for four of nine residues. In the latter instance, analysis according to Garnier *et al.* (1978) predicts mainly helical structure in this region, in which case the helix would be amphipathic (Figure 7). Fourthly, it is possible that the region between residues 564 and 585 contains a helix-turn-helix motif (Bennett, 1989). This region, as well as that containing the putative zinc finger, is basic (Figure 6). There is at present no evidence that a helix-turn-helix motif is involved in DNA binding by the *areA* product, however. Deletion of residues 561−597 eliminates *are*A function although this could be due to loss of five residues (561−565) from a region conserved with DNA binding domains of a vertebrate regulatory protein rather than to loss of a helix-turn-helix (See below).

Identification of non-essential regions of the areA gene

The translocation breakpoint region of an *are*A^r-18 strain was cloned using the inverse polymerase chain reaction (Ochman *et al.*, 1988) and sequenced directly. The position of the *are*A^r-18 translocation breakpoint is shown in Figure 3. This translocation is remarkable in that it can revert intracistronically (Arst *et al.*, 1989b). Intracistronic reversion of *are*A^r-18 occurs by additional chromosomal rearrangements associated with the 3′ moeity of the gene, presumably fusing a functional promoter, ribosome binding site and in-frame initiation codon to the rest of the gene (Arst *et al.*, 1989b). From the position of the *are*A^r-18 breakpoint, it is clear that intracistronic revertants will lack at least the 336 N-terminal amino acids. Thus the N-terminal 336 residues are either non-essential or can be replaced by sequences recruited from at least several other places in the genome. This conclusion is supported and extended by the *are*A$^+$ phenotype of transformants of an *are*A^r-18 strain in which a single copy of an in-frame deletion clone lacking a *Bst*EII fragment (sites shown in Figure 3) encoding residues 214−342 is integrated heterologously (summarized in Figure 8). In contrast, no *are*A$^+$ transformants have been obtained by either direct selection or cotransformation with selection for another marker using an in-frame *Sph*I deletion clone of *are*A lacking residues 135−474 (Figures 3 and 8).

The *are*A-associated *xpr*D-1 inversion truncates the 124

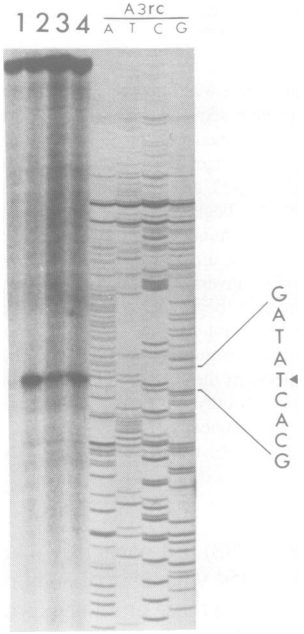


Fig. 4. S1 mapping of 5′ end of transcript. A 485 nucleotide single-stranded probe was synthesized by extension of oligonucleotide A3rc (TGATCCCTGCGGGCTT) using the single strand M13 clone XX1 as template and digestion at the *Bam*HI restriction site. The probe was hybridized to rRNA (**lane 1**) or polyadenylated RNA from a wild type strain grown for 16 h at 37°C on 100 μg/ml uric acid as sole nitrogen source (**lane 2**), 10 mM NH_4^+ as sole nitrogen source (**lane 3**) or 10 mM NH_4^+ as sole nitrogen source and then transferred to nitrogen-free medium for 2 h (**lane 4**). The sequencing ladder was made using the same oligonucleotide and template DNA as that used to synthesize the probe. The upper band in lanes 1−4 represents the undigested probe. The lower band in lanes 2, 3 and 4 represents the fragment protected by the *are*A transcript. This band correlates to a T (as indicated) in the untranslated strand. The equivalent A in the translated strand was confirmed as the 5′ start site in further experiments using probes with different 5′ and 3′ ends (not shown).

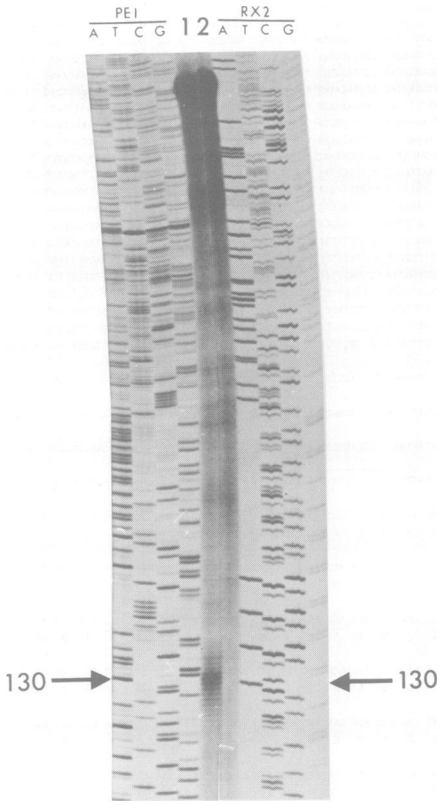


Fig. 5. S1 mapping end of 3′ end of transcript. A 342 nucleotide single-stranded probe was synthesized by extension of oligonucleotide RX4 RC (CCAGAAACTCCGCAGAT) with the single strand M13 clone EX as template and digestion at the *Kpn*I site. The probe was hybridized to polyadenylated RNA from wild type grown for 16 h at 37°C on 100 μg/ml uric acid as sole nitrogen source (**lane 1**) or rRNA (**lane 2**). Two separate sequencing ladders (PEI and RX2) were used as markers. A protected band of ~130 nucleotides is seen in lane 1 but not lane 2. A further experiment using a different probe gave a consistent result (not shown).

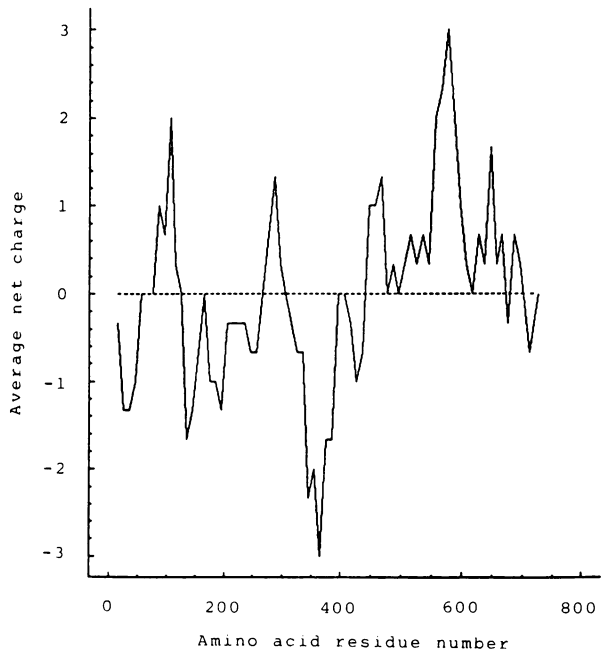


Fig. 6. Predicted charge distribution for the *are*A protein. The average net charge was calculated using a window of 30 amino acids at intervals of 10 amino acids using a program of Bennett (1989).

C-terminal residues from the *are*A protein (Figures 3 and 8). Although this inversion has little or no effect on the ability of the *are*A protein to activate gene expression, it does result in nitrogen metabolite derepression across the *are*A regulatory domain (Cohen, 1972; Arst and Cove, 1973; Pateman *et al.*, 1973). It is thus clear that the 124 C-terminal residues are not essential to the gene activation function of *are*A. However, the non-essential region at the C terminus cannot extend much further upstream: an in-frame deletion of residues 561–597 (Figures 3 and 8) did not complement *are*A^r-18 in cotransformation experiments.

Taken together these results indicate a maximum size for the essential region of *are*A of 253 residues, 35% of the protein comprising residues 343–595 (Figure 8). The bulk of the highly acidic region, the putative zinc finger and the possible helix-turn-helix all lie within this interval.

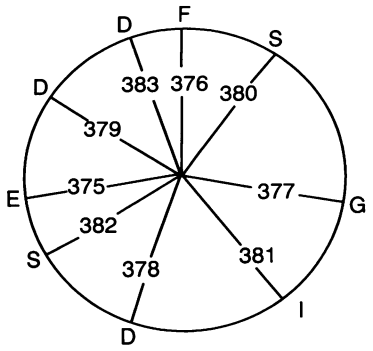


Fig. 7. Helical wheel analysis of residues 375–383. Note that all aspartate and glutamate residues are located on the left side of the helix as shown.

Identification of a region necessary for nitrogen metabolite repressibility

Nitrogen metabolite derepression associated with the *xpr*D-1 inversion has been noted above. The position of the inversion breakpoint suggests that a sequence in the 3′ part of the *are*A gene is involved in the ability of L-glutamine (and ammonium through its conversion to glutamine) to exert nitrogen metabolite repression. Supporting evidence for this conclusion as well as further localization of the sequence involved comes from properties of transformants in which a single copy of a 3′ deletion clone has integrated either heterologously or in the 5′ moeity of the translocation-split

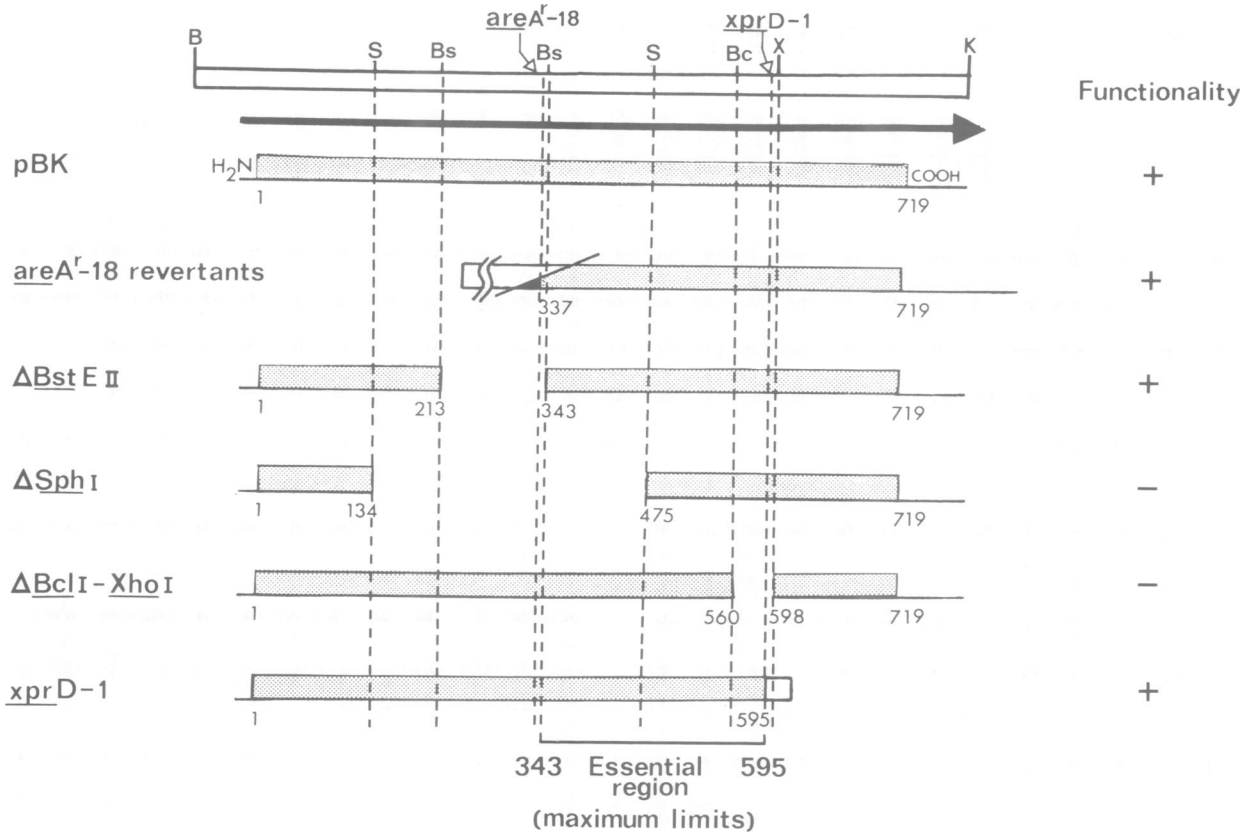


Fig. 8. Functionality of *are*A alleles lacking part of the *are*A gene though chromosomal rearrangements (*are*A^r-18 intracistronic revertants and *xpr*D-1) or transformation with a deletion clone (other alleles shown). An allele is defined as functional if it allows the utilization of nitrogen sources other than ammonium and glutamine. The positions of the *are*A^r-18 translocation and *xpr*D-1 inversion breakpoints and sites used for constructing *are*A deletion clones are shown in Figure 3. (B, *Bam*HI; Bc, *Bcl*I;. Bs, *Bst*EII; K, *Kpn*I; S, *Sph*I; X, *Xho*I). The pBK clone is shown in Figure 1. The Δ *Bst*EII, Δ *Sph*I and Δ *Bcl*I–*Xho*I clones are derived from pBK as described in Materials and methods. Single copy, heterologous transformants functionally *are*A$^+$ containing the pBK or Δ *Bst*EII clone were identified by Southern blotting. The lack of functionality of the Δ *Sph*I and Δ *Bcl*I–*Xho*I clones is based on lack of transformants in which the deletion is retained (see text). Further functional alleles truncated at the C terminus are shown in Figure 9 but are not shown here because they are truncated distal to the *xpr*D-1 breakpoint and therefore do not help to localize the essential region. Non-shaded and black bars indicate the presence of amino acid sequences foreign to *are*A. The precise localization of such fusion sequences in *are*A^r-18 revertants has not been done and the notation that normal *are*A sequence begins with residue 337 simply indicates that the *are*A^r-18 translocation removes residues 1–336. Additional chromosomal rearrangements associated with the reversion events might remove further N-terminal residues or fortuitously restore one or more N-terminal residues at the junction.

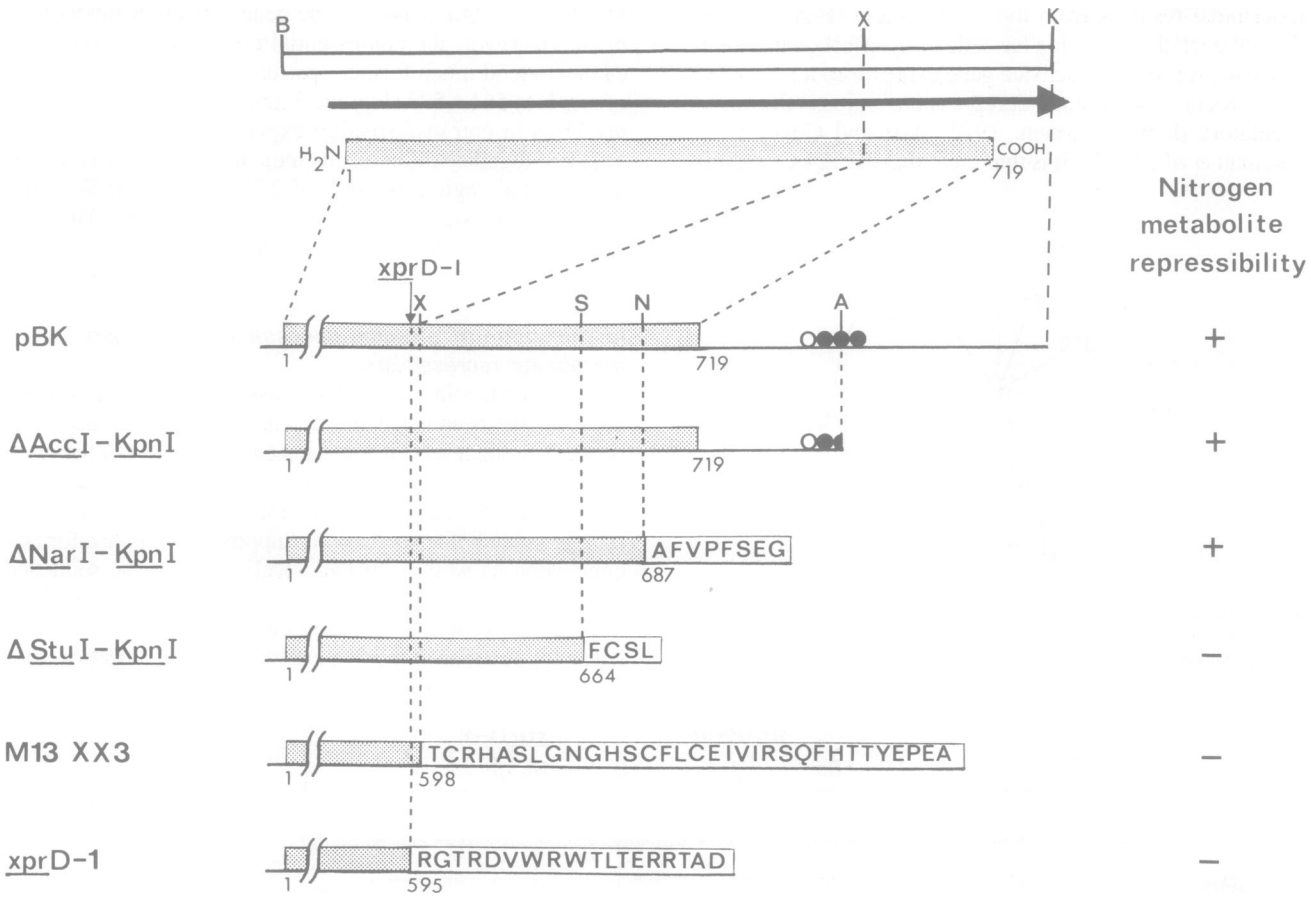


Fig. 9. Nitrogen metabolite repressibility and C termini of functional *are*A alleles associated with a chromosomal rearrangement (*xpr*D-1) or transformation with a deletion clone (other alleles shown). Positions of *xpr*D-1 inversion breakpont and restriction sites used for truncating the 3′ region of *are*A are shown in Figure 3. (A, *Acc*I;. B, *Bam*HI; K, *Kpn*I; N, *Nar*I; S, *Stu*I; X, *Xho*I). The pBK and M13 XX3 clones are shown in Figure 1. The Δ *Acc*I−*Kpn*I, Δ *Nar*I−*Kpn*I and Δ *Stu*I−*Kpn*I clones are derived from pBK as described in Materials and methods. Transformants used were identified by Southern blotting as containing a single copy of the deletion clone integrated heterologously or homologously in the 5′ moiety of an *are*A^r-18 recipient. (As the *are*A^r-18 translocation breakpoint lies upstream of all sites used to make deletions, homologous recombination in the 5′ moiety of the split *are*A^r-18 allele cannot restore any part of any deleted region.) Junction sequences of deletion clones were verified by sequencing. For *xpr*D-1 a clone crossing the inversion breakpoint was sequenced. Amino acid sequences are derived from DNA sequences done on both strands. Where junction vector sequences in a deletion clone result in the same amino acid as that of the wild type *are*A product, the resulting amino acids are included in the region of normal *are*A sequences. Filled circles indicate perfect copies of the 7 bp repeat, open circle for an imperfect copy—see Figure 3.

gene of an *are*A^r-18 recipient (such that no part of any deletion can be restored by recombination). The results are summarized in Figure 9. Truncation at the *Xho*I site, removing 121 C-terminal residues, or at the *Stu*I site, removing 55 C-terminal residues, results in a derepressed phenotype whereas truncation at the *Nar*I site, removing 32 C-terminal residues, or at the middle of the three *Acc*I sites, removing two of the three perfect copies of the 7 bp direct repeat from the 3′-untranslated region, still allows wild type repressibility. These results localize a sequence essential for repressibility within a region of 71 bp present in the *Nar*I deletion construct but absent from the *Stu*I deletion construct. Although the data do not establish whether nitrogen metabolite repressibility involves conformational changes of the *are*A protein rather than stability of its mRNA, it is intriguing to note that the *Stu*I truncation point lies within a 16 residue (beginning at 654) predicted α-helical region according to a Garnier *et al.* (1978) analysis. This predicted helix, having nine charged residues out of 16, is strongly basic at its N terminus and strongly acidic at its C terminus. The *Stu*I deletion clone lacks the six C-terminal residues of the predicted helix, which includes three of the four acidic residues.

Sequence similarities between the putative DNA binding region of the areA protein and those of a major DNA binding regulatory protein of vertebrate erythroid cell lineages

The gene specifying the major eyrthroid-specific transcription factor, designated variously GF-1, Eryf1 or NF-E1, regulating, *inter alia*, expression of the globin genes has been cloned and sequenced from several animals (Tsai *et al.*, 1989; Evans and Felsenfeld, 1989. Trainor *et al.*, 1990; Zon *et al.*, (1990). The derived protein sequences of these genes contain two putative zinc fingers of structure Cys-X_2-Cys-X_{17}-Cys-X_2-Cys separated by 29 residues. A 52 amino acid sequence from *are*A beginning at residue 514 and including the putative zinc finger plus 25 residues downsteam shows 63.5% identity in ungapped alignment with the corresponding C-terminal finger region of murine GF-1 in addition to several conservatively differing residues and reciprocal sequence relationships (Arst *et al.*, 1989a),

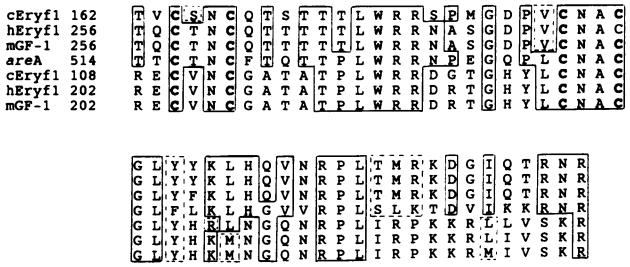


Fig. 10. Alignment of a 52 amino acid region of *are*A with two regions of *m*urine GF-1 (Tsai *et al.*, 1989), *c*hicken Eryf1 (Evans and Felsenfield, 1989) and *h*uman Eryf1 (Trainor *et al.*, 1990; Zon *et al.*, 1990). Residues identical to those of *are*A are boxed with unbroken lines and conservatively differing residues are boxed with broken lines.

Table I. Sequence changes resulting from *are*A mutations

Allele number	Nucleotide affected	Nucleotide change	Amino acid affected	Amino acid change
600	1466	C → A	489	Ser → TAG
102	1576	C → G	526	Leu → Val
30[a]	1576	G → A	526	Val → Met
31[a]	1576	G → A	526	Val → Met
19[b]	1601	A → C	534	Gln → Pro
209[b]	1615	G → C	539	Ala → Pro
217[b]	1622	G → A	541	Gly → Asp
12	1652	G → A	551	Arg → His
2	1792	−T	598	Ser → frameshift

[a]*are*A-30 and -31 were obtained by reversion of an *are*A-102 strain.
[b]*are*A^r-19, -209 and -217 were selected in *are*A-102 strains and the *are*A-102 mutation has been retained.

indicating very broad evolutionary conservation of this probable DNA binding region. Figure 10 compares, in ungapped alignment, this 52 amino acid region from *are*A with both N- and C-terminal finger sequences of the murine, human and chicken erythroid transcription factors, indicating both identical and conservatively differing residues. Not only is there a remarkable degree of sequence conservation between *are*A and the C-terminal finger regions of the eyrthroid factors but in a few cases where residues do differ there are sequence identities between *are*A and the N-terminal finger regions. One interpretation would be that duplication of the finger region now present in the eyrthroid factor predates the evolutionary divergence of animals and fungi. A closer evolutionary relationship between filamentous ascomycetes and animals than hitherto suspected has been proposed by Smith (1989).

areA mutant sequence changes

In addition to the *are*A^r-18 translocation and *xpr*D-1 inversion breakpoints, a number of sequence changes associated with point mutations in *are*A have been identified (Table I). To facilitate localization of mutant sequence changes a system for the fine-structure mapping of *are*A was developed (Arst, 1989 and unpublished). It compensates for the paucity of flanking markers and the lack of *are*A deletion mutations by using specificity/gain-of-function mutations as intragenic markers and exploiting the *are*A^r-18 translocation breakpoint. Fine-structure maping not only approximately localizes a mutatuon, it substantially increases the probability that an identified sequence change is solely responsible for an observed phenotype.

Loss-of-function mutations. Although allele-specific suppression in *A.nidulans* has been described (e.g. Bal *et al.*, 1978, 1979; Roberts *et al.*, 1979) and the suppressors further characterized (e.g. Bratt and Martinelli, 1988), *are*A^r-600 (Al Taho *et al.*, 1984) is the first such suppressible *A.nidulans* mutation where a sequence change to a chain termination codon (amber in this case) has been demonstrated. It is also worth noting that the *are*A^r-600 sequence change confirms the reading frame at that point: in the other two reading frames there would be no change in amino acid sequence. Arguably the most interesting loss-of-function mutation to be characterized here is *are*A^r-2, a −1 frameshift mutation destroying the *Xho*I site within *are*A and eliminating the 122 C-terminal amino acids. At first sight this result is paradoxical in view of the evidence (see above) that the 124 C-terminal residues are not essential. Scrutiny of the consequences of the frameshift provides a possible explanation: the 122 C-terminal residues are replaced by a peptide of 117 amino acids of sequence RAGPKAIR-HPSLPQQCRVAPAEALESFLLRLPRLNLAQRRLRL-RAQTTVVVRYRWLLNVSVGWKRPLMLIWRKARP-VPRPVDDPRLFHWRRLCLQRPSTQPTTVSLLVRAQ-ARNGSG. Whereas the C-terminal 122 residues of the wild type *are*A protein include ten basic and seven acidic amino acids, the 117 C-terminal residues in an *are*A^r-2 strain include 24 basic and four acidic amino acids, giving the mutant C terminus a higher net positive charge than the putative DNA binding region. In contrast none of the foreign C termini of functional C-terminal truncation genes (Figure 9) carries a high net charge. Whilst there might be other reasons why the 117 residue C terminus of *are*A^r-2 eliminates *are*A function, its +20 net charge must surely be the most obvious candidate.

A further four loss-of-function mutant sequence changes occur within the 52 residue region conserved between *are*A and the erythroid transcription factor. It is hardly surprising that *are*A^r-19 and -209 result in loss of function as replacement of other residues by proline in the finger itself would be expected to have major conformational consequences and therefore profound effects on DNA binding. Both *are*A^r-217 and -12 (as well as *are*A^r-209) result in replacement of residues conserved throughout all seven finger regions of this class (Figure 10). In addition both *are*A^r-217 and -12 reduce the basicity of this strongly basic region.

Mutations affecting specificity. Clearly the most interesting of the mutant sequence changes are those associated with specificity mutations having variously a loss-of-function, gain-of-function or wild type phenotype depending upon the structural gene whose expression is being monitored. The *are*A-102 mutation has been extensively described (Hynes, 1973a,b; 1975; Arst and Cove, 1973; Arst and Scazzocchio, 1975; Polkinghorne and Hynes, 1975; Arst, 1977; Gorton, 1983). It leads to elevated expression of a number of activities including acetamidase, histidase and permeases for urea, L-glutamate and L-citrulline and to reduced expression of certain other activities including formamidase and xanthine-uric acid permease. L.Gorfinkiel, G.Diallinas and C.Scazzocchio (unpublished results) have demonstrated an absence of mRNA for the xanthine-uric acid permease encoded by *uap*A (Diallinas and Scazzocchio, 1989) in an *are*A-102 strain. The *are*A-30 and -31 mutations (Arst and

Scazzocchio, 1975, 1985; Gorton, 1983) were obtained by reversion of an *are*A-102 strain and their (identical) phenotypes are in part reciprocal to or a mirror image of that of *are*A-102. For example, whereas *are*A-102 strains grow more strongly than wild type strains when utilizing acetamide, L-glutamate or L-aspartate as nitrogen source, due to elevated acetamidase and acidic amino acid permease levels, *are*A-30 and -31 strains utilize these nitrogen sources more poorly than wild type. Whereas *are*A-102 strains fail to utilize xanthine and uric acid as nitrogen sources and are resistant to the toxicities of their analogues (detailed by Darlington and Scazzocchio, 1967 and Arst and Scazzocchio, 1975) as a result of reduced uptake, *are*A-30 and -31 strains grow more strongly than wild type on xanthine and uric acid and are hypersensitive to analogue toxicities, correlating with elevated uptake (Arst and Scazzocchio, 1975; Gorton, 1983). For urea uptake the situation is reversed: *are*A-30 and -31 strains utilize urea very poorly, are resistant to the toxic analogue thiourea and have reduced uptake levels whereas *are*A-102 strains grow more strongly than wild type on urea and are hypersensitive to thiourea (Hynes, 1973a; Gorton, 1983). The *are*A-102 mutation results in a leucine to valine change in a completely conserved residue (Figure 10) of the central portion of the finger loop whilst *are*A-30 and -31 are identical changes resulting in a methionine residue in the same loop position. It is thus clear that residues in the loop of the putative zinc finger of *are*A can play an important role in the recognition of *are*A receptor sites. Interestingly a possibly comparable specificity mutation in the yeast regulatory gene *CYP1* (*HAP1*) alters a residue adjacent to a zinc finger (Verdière *et al.*, 1988; Kim and Guarente, 1989 and references therein). Moreover, a central residue of a zinc finger loop is clearly important in the recognition of receptor sites for the yeast regulatory protein GAL4 (Corton and Johnston, 1989). The remarkable diversity of *are*A mutant phenotypes should enable the *are*A system to make substantial further contributions to an understanding of the determinants of specificity of structural gene activation.

Materials and methods

A.nidulans strains, culture and genetic techniques

A.nidulans strains carried markers in standard use (Clutterbuck, 1987). The *xpr*D-1, *are*A-102 and *are*A^r-2, -18, -19, -209, -217 and -600 mutations have been described previously (Cohen, 1972.; Arst and Cove, 1973; Hynes, 1973a, 1975; Rand and Arst, 1977; Arst, 1981, 1982; Al Taho *et al.*, 1984; Caddick *et al.*, 1986; Arst *et al.*, 1989b). The *are*A^r-12 mutation was induced by diethylsulphate and selected, using replica plating, as able to utilize ammonium but not hypoxanthine as nitrogen source in a biotin-requiring strain of genotype *bi*A-1 (C.Scazzocchio and A.J.Darlington, unpublished). The *are*A-30 and -31 mutations were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis in a strain of genotype *bi*A-1 *uap*A-24 *are*A-102 *fw*A-1 (requiring biotin, lacking one of the xanthine-uric acid permeases, carrying the specificity mutation *are*A-102, having fawn conidial colour) and selected as allowing utilization of 100 μg/ml xanthine and uric acid, respectively. The basis for the selection depends on the fact that *are*A-102 results in lack of expression of both *uap*A (Darlington and Scazzocchio, 1967; Arst and Scazzocchio, 1975; Gorton, 1983.; Diallinas and Scazzocchio, 1989) and at least one other permease for xanthine and uric acid. The (phenotypically indistinguishable) *are*A-30 and -31 mutations result in regain with overexpression of both *uap*A function and that of another xanthine-uric acid permease (Gorton, 1983). Because the parental strain carries the loss-of-function mutation *uap*A-24, regain (and probably overexpression) of the alternative xanthine-uric acid permease(s) was probably crucial to recovery of the *are*A-30 and -31 mutations.

Standard genetic techniques were used (Pontecorvo *et al.*, 1953; Clutterbuck, 1974). Growth media and culture procedures followed Cove (1966). Growth testing of *A.nidulans* has been described previously (Arst and Cove, 1969, 1973. Arst *et al.*, 1982). Growth tests were carried out at 37°C in minimal media containing 1% (w/v) D-glucose as carbon source. To be classified as nitrogen metabolite derepressed (i.e. '−' for repressibility in Figure 9) transformant and chromosomal rearrangement strains have met five criteria: (i) sensitive to 100 mM chlorate toxicitiy with 10 mM ammonium as nitrogen source, indicating derepression of nitrate reductase (Arst and Cove, 1969). (ii) sensitive to 5 mM DL-β-aspartylhydroxamate toxicity on 10 mM ammonium as nitrogen source, indicating derepression of an asparaginase (Drainas *et al.*, 1977; Shaffer *et al.*, 1988); (iii) clearing the turbidity to produce a halo on medium containing 0.65% (w/v) powdered skimmed milk and 10 mM ammonium, indicating derepression of extracellular protease (Cohen, 1972), (iv) reducing 10 mM nitrite in the presence of 10 mM ammonium such that proton depletion allows 0.025% (w/v) bromothymol blue in the growth medium to remain blue/green (as opposed to wild type yellow due to pH drop), indicating derepression of nitrite reductase (Rand, 1978); (v) sensitive to 100 μg/ml 2-thioxanthine toxicity with 10 mM ammonium as nitrogen source, indicating derepression of xanthine-uric acid permease and xanthine dehydrogenase (Darlington and Scazzocchio, 1967.; Arst and Cove, 1969; Arst and Scazzocchio, 1975).

Nucleic acid preparation, electrophoresis and Northern blotting

Bacteriophage and plasmid DNAs were prepared by standard procedures (Maniatis *et al.*, 1982; Messing, 1983). *A.nidulans* genomic DNA was prepared by the method of Raeder and Broda (1985) except that 2% sarkosyl was used in place of SDS in the extraction buffer. RNA was prepared by the method of Clements and Roberts (1985). Polyadenylated RNA was selected using oligo(dT) cellulose (Sigma) by standard procedures (Maniatis *et al.*, 1982). For Northern blots 10 μg of polyadenylated RNA was electrophoresed on formaldehyde gels by the method of Colman (1984). RNA mol. wt markers (Boehringer-Mannheim) and *A.nidulans* rRNA (Bartnik and Borsuk, 1983) were used to estimate transcript size. The RNA was transferred to Hybond-N (Amersham) membranes. Hybridization was carried out at 65°C for 16 h in a solution with a final concentration of 5 × SSC, 10 mM EDTA, 50 mM PO^{3-}_4 (pH 6.5), 5 × Denhardt's, 0.5% (w/v) SDS and 100 μg/ml sonicated salmon sperm DNA. The filters were washed at 65°C in 0.2 × SSC containing 0.1% SDS. Double stranded probes were prepared by the method of Feinberg and Vogelstein (1984).

Subcloning and sequencing

Subcloning of the 11.6 kb λ Charon 4 clone 16.7 (Figure 1) followed standard procedures (Maniatis *et al.*, 1982). M13 XX1 and M13 XX3 were constructed by ligating the 2.1 kb *Xho*I fragment into the *Sal*I site of M13mp18 (Yanisch-Perron *et al.*, 1985). M13 XX1 contains the positive and M13 XX3 the negative strand. pEB was constructed by ligating the 4.8 kb *Eco*RI−*Bam*HI fragment into *Eco*RI-, *Bam*HI-digested pBR325 (Prentki *et al.*, 1981). M13 EX and M13 XE were constructed by cloning the 2.8 kb *Eco*RI−*Xho*I fragment into the *Eco*RI and *Sal*I sites of M13mp19 and -mp18, respectively. pBK was constructed by cloning the 2.8 kb *Bam*HI−*Kpn*I fragment into *Bam*HI-, *Kpn*I-digested Bluescript KS$^+$ (Stratagene). pKK was constructed by cloning the 4.5 kb *Kpn*I fragment into the *Kpn*I site of pUC19 (Yanisch-Perron *et al.*, 1985). M13 BK and M13 KB were constructed by ligating the 2.8 kb *Bam*HI−*Kpn*I fragment into *Bam*HI-, *Kpn*I-digested M13mp18 and -mp19, respectively.

*are*A deletion clones were constructed from pBK. Internal deletions were constructed by digestion with *Bst*EII, *Sph*I or *Bcl*I plus *Xho*I as appropriate (Figure 8) followed by religation. In the case of the *Bcl*I−*Xho*I deletion, pBK DNA was prepared from a dam3$^-$ mutant of GM33 F$^-$ W3110 obtained from M.Radman, Institut Jacques Monod, Paris; after digestion with *Bcl*I and *Xho*I the protruding ends were filled using Klenow enzyme and the four dNTPs. The product was then religated. 3′ deletions involved digestion with *Kpn*I and *Acc*I, *Nar*I or *Stu*I as appropriate (Figure 9). The protruding ends were removed with mung bean nuclease (Pharmacia) and the product religated. The *Nar*I and *Acc*I deletions were constructed from the *Sph*I internal deletion clone and the *Bam*HI−*Xho*I fragment was subsequently replaced by that of pBK. All the constructs were checked by sequencing.

The pBK clone and its derivatives were co-transformed into an *are*A^r-18 *arg*B-2 recipient strain with the *arg*B$^+$-containing plasmid pILJ16 (Johnstone *et al.*, 1985) by the procedure of Ballance and Turner (1985). Arginine-independent transformants were selected and scored for *are*A phenotype. In the case of the *Sph*I internal deletion clone, direct selection of *are*A$^+$ transformants on nitrate as nitrogen source was also attempted. For the *Bcl*I−*Xho*I deletion, three out of 50 transformants selected as *arg*B$^+$ were also phenotypically *are*A$^+$. However, Southern blotting showed that in all three cases the deletion *are*A clone had recombined homologously in the 3′ moiety of *are*A upstream of the *Bcl*I site, thus recreating a wild type gene (data not shown). (If the *Bcl*I−*Xho*I deletion

clone were capable itself of complementing *are*A^r-18, it would be expected that the number of homologous recombination events elsewhere in *are*A plus heterologous recombination events giving an *are*A$^+$ phenotype would greatly exceed the number of homologous recombination events in the 673 bp interval between the *are*A^r-18 breakpoint and the *Bcl*I cleavage site. As three of the latter but none of the former were obtained, it is extremely likely that deletion of the *Bcl*I–*Xho*I fragment removes an essential residue(s). M13 XX3 was transformed into an *are*A^r-18 recipient strain and tranformants selected directly for their ability to grow on 10 mM NO_3^- as sole nitrogen source. All transformants were characterized by Southern hybridization analysis. The DNA sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Sequenase II, USB). Nucleotide sequencing of both strands was completed with synthetic oligonucleotides using M13 XX1, M13 XX3, pBK (with insert in both orientations), M13 EX and M13 XE clones and their derivatives as single-stranded template DNA. Band compression was resolved using deoxyinosine triphosphate (dITP) in place of dGTP.

Nuclease protection experiments

Single strand DNA probe synthesis was based on the method of Burke (1984). 1 μg of single-stranded template DNA, 3 μg of primer and 1 μl of TM (100 mM Tris–HCl, 50 mM $MgCl_2$, pH 8.5) was made up to 10 μl with H_2O. The solution was heated to 65°C for 2 min and allowed to cool slowly to 37°C. To this was added 1 μl of 4 nM dCTP, 1 μl each of 2 mM dATP, dGTP and dTTP, 25 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 1–2 units of Klenow fragment and H_2O to a final volume of 20 μl. After incubation for 20 min at 20°C, 1 μl of 2 mM dCTP was added and the solution incubated for a further 20 min. The Klenow enzyme was inactivated by addition of 1 μl of 3 mM EDTA and incubation at 65°C for 10 min. The DNA was then digested with the required restriction enzyme in a final volume of 30 μl, denatured by the addition of 3 μl of 4 M NaOH for 2 min at room temperature. The single-stranded DNA probe was located by autoradiography. The appropriate region of the gel was excised, and the DNA extracted using Geneclean (BIO 101). The DNA was phenol-extracted and then co-precipitated with the apropriate RNA. Nuclease protection followed the method of Calzone *et al.*, (1987). Each nuclease digest utilized 1000 units of S1 nuclease (Boehringer-Mannheim) at 18°C for 1 h. The amount of wild type RNA used in each reaction was: 20 μg rRNA, 10 μg polyadenylated RNA after growth on NH_4^+ as sole nitrogen source, or 2 μg polyadenylated RNA after growth on uric acid as sole nitrogen source, or nitrogen-starved for 2 h. Half the final product was electrophoresed on standard wedge polyacrylamide sequencing gels with a sequencing ladder to determine the size of the product. The gels were autoradiographed at −70°C for 16 h.

Cloning and sequencing of point mutant areA alleles

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) was used to amplify *are*A sequences from *are*A^r-2, -12, -19, -209 and -217 and *are*A-30, -31 and -102 strains. PCR was carried out in 100 μl final volume, containing 0.1 μg of chromosomal DNA from the appropriate mutant strain. The reaction buffer had a final concentration of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$ 0.01% (w/v) gelatin, 200 μM each of dNTP, 1 μM of two appropriate primers (17–25 nucleotides long), and 2.5 units of Taq polymerase (Perkin-Elmer Cetus). Generally 30 cycles of 45 s at 90°C, 1–2 min at 37°C and 1–3 min at 72°C were conducted by hand. 5 μl of the product was examined by gel electrophoresis and Southern blotting. The remainder of the sample was then electrophoresed separately and the appropriate fragment excised. DNA was extracted and purified using Geneclean (BIO 101). The product was made up of a final volume of 20 μl with H_2O, placed at 100°C for 3 min in an open Eppendorf tube, and then transferred to wet ice. This denatured DNA was then used as the substrate for sequencing with Sequenase II (USB). Oligonucleotides were annealed at 37°C for 20 min; otherwise the standard reaction protocol was employed except that 'labelling mix' was diluted 1:15. Mutations were confirmed by sequencing both strands.

Cloning areA^r-18

Using restriction analysis and Southern blotting of genomic DNA the *are*A^r-18 translocation breakpoint was shown to lie between an *Apa*I site (Arst *et al.*, 1989b) and a *Bst*EII site (sites shown in Figure 3). In order to clone across the breakpoint, inverse PCR (Ochman *et al.*, 1988) was employed. Whereas the wild type *Apa*I fragment overlapping the *are*A^r-18 translocation breakpoint contains 859 bp (Arst *et al.*, 1989b), the *are*A^r-18 translocation fuses the 3′ part of the *Apa*I fragment to a chromosome IV sequence such that a 2.1 kb *Apa*I fragment is created. 2 μg of *are*A^r-18 genomic DNA was digested to completion with *Apa*I and electrophoresed. The appropriate region of the gel was excised and the DNA extracted using Geneclean (BIO 101). The purified DNA was circularized by ligation at low concentration (1 ml final volume) at 18°C for 16 h. The ligation product was phenol-extracted, ethanol-precipitated and redissolved in 50 μl H_2O. 5 μl of the DNA solution was used as substrate for PCR utilizing two oligonucleotides which would prime divergent DNA synthesis in the wild type. This amplified a region which included the *are*A^r-18 breakpoint. The PCR product was sequenced directly.

Cloning xprD-1 and areA-102

Southern hybridization analysis was used to identify suitable restriction enzyme sites for digestion and cloning. Size-selected *Eco*RI-digested genomic DNA was cloned into EMBL lambda vectors (Frishchauf *et al.*, 1983). The resulting gene libraries were plated and screened using *are*A-specific probes, as described previously (Caddick *et al.*, 1986. Bennett, 1989). A clone crossing the *xpr*D-1 inversion breakpoint was subcloned into M13mp18 and -mp19 and an *are*A-102-containing clone was subcloned into Bluescript KS$^+$. Both alleles were sequenced in both orientations. The *are*A-102 sequence change was additionally confirmed by sequencing PCR-amplified genomic DNA as described above.

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Note added in proof

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X52491. Additional evidence for the importance of the 'finger' is that a newly sequenced null mutation converts Cys-519 to Arg. The presence of a TPXX motif (TPLW) within the 'finger' should be noted as should the occurrence of the *are*A^{r}-30, -31 and -102 sequence changes within this motif. The sequence of the corresponding *nit*2 gene of *Neurospora crassa* has been reported [Fu and Marzluf, *Mol. Cell Biol.*, **10**, 1056–1065 (1990)]. Sequence relationships will be detailed elsewhere. A role for duplication in the evolution of the *nit*2 sequence has been noted (*Ibid.*) This is also apparent in *are*A when amino acid (or nucleotide) sequences beginning with Ser-616 are compared with those beginning with Ser-676: 10 of 16 residues are identical, one is conservatively changed, and the order of two others is reversed.