Chimeric purine transporters of *Aspergillus nidulans* define a domain critical for function and specificity conserved in bacterial, plant and metazoan homologues

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In *Aspergillus nidulans*, purine uptake is mediated by three transporter proteins: UapA, UapC and AzgA. UapA and UapC have partially overlapping functions, are 62% identical and have nearly identical predicted topologies. Their structural similarity is associated with overlapping substrate specificities; UapA is a high-affinity, high-capacity specific xanthine/uric acid transporter. UapC is a low/moderate-capacity general purine transporter. We constructed and characterized UapA/UapC, UapC/UapA and UapA/UapC/UapA chimeric proteins and UapA point mutations. The region including residues 378–446 in UapA (336–404 in UapC) has been shown to be critical for purine recognition and transport. Within this region, we identified: (i) one amino acid residue (A404) important for transporter function but probably not for specificity and two residues (E412 and R414) important for UapA function and specificity; and (ii) a sequence, (F/Y/S)X(Q/E/P)NXGXXXXT(K/R/G), which is highly conserved in all homologues of nucleobase transporters from bacteria to man. The UapC/UapA series of chimeras behaves in a linear pattern and leads to an univocal assignment of functional domains while the analysis of the reciprocal and 'sandwich' chimeras revealed unexpected inter-domain interactions. cDNAs coding for transporters including the specificity region defined by these studies have been identified for the first time in the human and *Caenorhabditis elegans* databases.

Keywords: *Aspergillus nidulans*/chimeric genes/mutation analysis/nucleobase transport

1968; Vogels and van der Drift, 1976; Scazzocchio, 1994). In plants and animals, purine transporters seem to be expressed ubiquitously allowing purine redistribution in specialized tissues which are unable to synthesize purines *de novo* (Fox and Kelley, 1978; Griffith and Jarvis, 1993; Frommer *et al.*, 1994; Sadee *et al.*, 1995; Washington and Giacomini, 1995). Uric acid, the final product of purine catabolism in primates, is excreted through a complex process involving specific transporters along the renal pathway (Abramson and Lipkowitz, 1990). Very recently a rat uricase-like protein that functions as a specific urate channel was described. This is a facilitated diffusion channel that does not belong to the class of transporters described below (Leal-Pinto *et al.*, 1997). In spite of their general physiological importance and the utilization of purine and pyrimidine analogues as anti-tumor and antiviral agents (Sadee *et al.*, 1995), nothing is known about the mechanism underlying the specificity of purine transport. In this article we present results obtained using an eukaryotic model system, the ascomycete *Aspergillus nidulans*.

In *A.nidulans*, three major purine transporters are encoded by the genes *uapA*, *azgA* and *uapC* (Scazzocchio, 1994; Diallinas *et al.*, 1995). The UapA protein is a high-affinity, high-capacity transporter specific for uric acid and xanthine, their thioanalogues (2-thiouric acid and 2-thioxanthine), allopurinol and oxypurinol. The AzgA protein is a high-affinity, high-capacity transporter specific for adenine, guanine and hypoxanthine, and the analogues 8-azaguanine and purine. Finally, the UapC protein is a wide-specificity, low/moderate-capacity carrier transporting all natural purines as well as purine analogues (Darlington and Scazzocchio, 1967; Arst and Scazzocchio, 1975; Scazzocchio and Gorton, 1977; Diallinas and Scazzocchio, 1989; Gorfinkiel *et al.*, 1993; Diallinas *et al.*, 1995). The UapC-dependent uptake is also cryosensitive and pH sensitive (Diallinas *et al.*, 1995; G.Diallinas, C.Scazzocchio, L.Gorfinkiel and H.N.Arst,Jr, unpublished observations). We have recently cloned, sequenced and studied in detail the transcriptional regulation of the *uapA* and *uapC* genes (Diallinas and Scazzocchio, 1989; Gorfinkiel *et al.*, 1993; Diallinas *et al.*, 1995; Ravagnani *et al.*, 1997). The UapA and UapC transporters share 62% identical amino acids throughout their amino acid sequences and belong to a distinct family of purine-pyrimidine carriers conserved in both prokaryotes and eukaryotes (Diallinas *et al.*, 1995; Guimarães *et al.*, 1995; Schultes *et al.*, 1996). Members of this transporter family share 25–65% identical amino acid residues and a common structural motif of 12–14 α -helical, transmembrane domains (TMs). Interestingly, the genome of *Saccharomyces cerevisiae* does not include any gene encoding similar transporters. In this article we analyse structure–function relationships in the UapA and UapC proteins.

Introduction

Purine transport is a widespread phenomenon in prokaryotes and eukaryotes. Many microorganisms are able to use external purines in their salvage pathways and/or as secondary nitrogen sources (Scazzocchio and Darlington,

We have approached this problem by constructing chimeras between these proteins, and by analysis of point mutations in regions defined as important by the chimeric protein analysis. These studies have led to the identification of a short region and a number of specific amino acid residues critical for purine transporter function and specificity.

Results

Construction and functional analysis of chimeric UapC/UapA (CA) transporters

To eliminate experimental bias in the positioning of chimeric junctions and simplify experimental procedures, chimeric transporters were constructed *in vivo* in *Escherichia coli* cells (Moore and Blakely, 1994; Nishizawa *et al.*, 1995) as described in the Materials and methods.

To obtain *uapC/uapA* chimeric genes, we first constructed plasmid pAN950. This plasmid carries the *uapC* and *uapA* genes in tandem, *argB* for selection in *A.nidulans* (growth in the absence of arginine) and *bla* for selection in *E.coli* (ampicillin resistance). *Escherichia coli* cells were transformed with the linearized plasmid (see Materials and methods). Restriction analysis of random clones obtained in independent experiments showed that 10–15% of transformants arose from recombination of *uapC* and *uapA* sequences resulting in chimeric *uapC/uapA* genes (data not shown). DNA sequencing showed that in-frame recombination occurred in a variety of points within the *uapC* and *uapA* genes (Figures 1 and 2A). Recombination events involved regions where an identity of at least 10 bp exist between *uapA* and *uapC* DNA sequences (data not shown).

Seven selected plasmids containing chimeric *uapC/uapA* (CA) genes called CA-1, -2, -3, -4, -5, -6 and -7 (Figures 1 and 2A), and control plasmids pAN510 and pAN910 carrying *uapA* and *uapC*, respectively (for detailed description of plasmids see Materials and methods) were introduced by transformation in a *uapA*⁻*uapC*⁻*azgA*⁻*argB*⁻ strain. Transformants were selected on minimal media without arginine. For each of the chimeric and the control plasmids, several transformants were selected and purified by their ability to grow on arginine-free medium. Transformants were analysed by Southern blots (data not shown). Further work, in this section and throughout the work described in this article, was carried out exclusively with transformants resulting from single homologous integration events at the *argB* locus.

All selected transformants were tested for utilization of hypoxanthine, adenine, xanthine and uric acid as sole nitrogen sources at 25 and 37°C at pH 6.8. Utilization of uric acid was also checked over a range of pHs (5–9.5) at 25 and 37°C. It appeared that while the transformant carrying the *uapA*⁺ (pAN510) plasmid behaved exactly as an *uapA*⁺*uapC*⁻*azgA*⁻ strain constructed by classical genetics and carrying an *uapA* wild-type allele, transformants carrying the *uapC*⁺ (pAN910) plasmid did not behave exactly like the *uapA*⁻*uapC*⁺*azgA*⁻ strain (Diallinas *et al.*, 1995). The transformed *uapC*⁺ strains show weaker growth on hypoxanthine, and reduced cryosensitivity and sensitivity to alkaline pHs than the *uapA*⁻*uapC*⁺*azgA*⁻ strains constructed previously by classical genetics (Diallinas *et al.*, 1995). This difference was found in all

Fig. 1. Amino acid sequence alignment of UapA and UapC showing the junctions (vertical lines in bold) of CA and AC chimeras. The alignment was created by the program GCG/GAP; vertical lines indicate identical amino acids, double or single dots indicate similar amino acids. Gaps are introduced by the program to maximise similarity scores. The cut-points of the different CA and AC chimeric proteins are indicated. For the sake of simplicity, arbitrary cut-points rather than boundaries are indicated, since the exact, actual cut-point could be anywhere within the limited region of complete nucleotide identity (see text). Different cut-points within regions of total amino acid identity (e.g. AC-3 and CA-2) can be established as the identity at the nucleotide level is not complete.

single-copy transformants, and also when a different *uapC*⁻ allele was present in the recipient strain (Diallinas *et al.*, 1995). It is possible that there is a polymorphism at the *uapC* locus and that our laboratory strains carry a different *uapC* allele to that present in the cloned phage (isolated from an EMBL4 library constructed by C.Lazarus). In this work we have used the allele defined by the sequence published by Diallinas *et al.* (1995) as a wild-type control for all experiments, and this sequence is contained in the transforming plasmids and in the original EMBL4 clone. As detailed below, results are internally consistent, as all transformants carrying crucial portions of the *uapC* gene behave exactly like the *uapA*⁻*uapC*⁺*azgA*⁻ strain obtained by transformation with the *uapC*⁺ plasmid, rather than as *uapC*⁺ strains constructed previously by crossing.

In growth tests, transformants expressing chimeric proteins CA-1, -2, -3, -4 and -5 behave exactly like the *uapA*⁺ control. These transformants carry chimeras comprising at most 335 residues derived from UapC in their N-terminus and at least 238 residues derived from UapA in their C-terminus (Figure 1). They grow strongly on uric acid, show no sensitivity to pH 7.4 and show no residual growth on hypoxanthine. Transformants carrying CA-6 and -7,

Fig. 2. Predicted topology of UapA, UapC, and CA (A), and AC (B) chimeric proteins. A 14 α -helical topology was predicted according to a program based on multiple sequence alignment of homologous proteins (Person and Argos, 1994). Putative TMs of 19–21 amino acids are shown as cylinders connected to each other by lines corresponding to polar regions of various lengths. The membrane environment is shown as a dotted black bar. The extracellular compartment is shown above, and the intracellular compartment is shown below the protein. Notional positions of chimeric junctions are shown (see legend to Figure 1); UapC-derived regions are indicated as white cylinders connected with standard lines while UapA-derived regions are indicated as grey cylinders connected with bold lines. Mirror-image CA/AC chimeras are drawn side by side.

i.e. chimeras where the break point occurred after residue 455 of UapA, behave exactly as the strains transformed with the *uapC*⁺ plasmid. These strains grow slightly better than the recipient strain on hypoxanthine, and show the mild sensitivity to growth at low temperatures and at $\geq$ pH 7.4 (tested between pH 7.4 and 9.5), typical of strains transformed with a non-chimeric (pAN910) *uapC* gene. Examples of phenotypes on uric acid are shown in Figure 3.

Table I shows that: (i) all transformants expressing chimeric transporters CA-1, -2, -3, -4 and -5 (Figures 1 and 2A) exhibit UapA-like transport activities (indistinguishable from those shown by transformants carrying a non-chimeric UapA plasmid, pAN510); (ii) all transformants obtained with the two most extreme C-terminal chimeras (CA-6 and -7; Figures 1 and 2A), exhibit UapC-like transport. Uric acid, xanthine and hypoxanthine are transported at rates indistinguishable from those found in transformants obtained with a non-chimeric *uapC* plasmid

(pAN910). There is a perfect agreement between the growth phenotypes and the uptake studies.

Construction and functional analysis of chimeric UapA/UapC (AC) transporters

The phenotypes of the CA chimeric proteins define a short region (368–463 in UapA, 326–421 in UapC) as a critical determinant of the specificity of purine transport. As this sequence is bound by regions of total identity (Figure 1), the region determining substrate specificity is reduced to amino acid residues 378–446 in UapA and 336–404 in UapC. To test further the functional importance of this region, we constructed a series of reciprocal chimeric proteins by inverting the order of the *uapA* and *uapC* gene segments. pAN590, a plasmid carrying the *uapA* and *uapC* genes in tandem, was constructed, linearized and used to select *uapA/uapC* (AC) chimeric genes in *E. coli*, as described above (see Materials and methods for details). After restriction analysis and DNA sequencing, five

chimeric genes, called AC-1, -2, -3, -4 and -5 (Figures 1 and 2B), were selected and introduced to *A.nidulans* as previously described. Three of them encoded nearly exact

Fig. 3. Growth of chimeras on uric acid as sole nitrogen source. ‘-’ indicates the triple mutant *uapA⁻uapC⁻azgA⁻*. This is the recipient strain described in the text (*argB⁻uapA⁻uapC⁻azgA⁻*) carrying a single homologous integration of plasmid pIJL16 which includes the *argB⁺* gene. ‘+’ indicates a wild-type strain *uapA⁺uapC⁺AzgA⁺*. UapC is the recipient strain carrying a single copy of *uapC⁺*. *uapA* is the recipient strain carrying a single copy of *uapA⁺*. E412Q/R414E indicates the double mutant of *uapA* as described in the text. All other strains are representative chimeric strains described in the text and in Figure 2. All strains are *argB⁺* as they carry the relevant construction integrated in one copy in the *argB* locus as described in the text. All strains carry in addition the *yA2* (yellow conidiospores) marker. In addition the wild-type strain is *pabaA1* (auxotroph for *p*-aminobenzoic acid). The medium is *Aspergillus* minimal medium with 600 mM uric acid as sole nitrogen source supplemented with *p*-aminobenzoic acid. All strains grow equally well on control media [not shown: urea, sodium nitrate or ammonium D(+) tartrate as sole nitrogen sources]. Growth tests were carried out for 48 h at 37°C and 4 days at 25°C.

mirror-image chimeric transporters of previously described CA chimeras; chimera AC-1 corresponds to CA-1, AC-3 to CA-2 and AC-5 to CA-6. AC-4 is a chimera with a boundary between those present in CA-3 or CA-4 (for comparison see Figures 1 and 2). *Aspergillus nidulans* transformants were selected and analysed as previously described. Southern blot analysis showed that all integration events occurred at the *argB* locus (data not shown). Single-copy transformants were obtained with clones AC-3, -2, -5 and -4. All AC-1 transformants were the result of multiple integration events and were not analysed further. Transformants carrying AC-2, -3 and -4 grew very poorly on uric acid (or xanthine) and do not utilize hypoxanthine as nitrogen source. In fact, the growth is hardly better than that of the recipient strain. AC-5 showed an entirely new phenotype; it grew as well as a *uapC⁺* control on uric acid at 37°C, showed no residual growth on hypoxanthine and showed extreme cryosensitivity for growth on uric acid. The cryosensitivity of strains carrying this chimera on uric acid is in fact not only greater than that of the control strain, but also more extreme than that of *uapC⁺azgA⁻uapA⁻* strains constructed by conventional crosses of our laboratory strains. Examples of growth phenotypes on uric acid are shown in Figure 3.

Uptake studies with AC-5 are completely consistent with the growth tests; it takes up uric acid and xanthine at a rate similar to that of the *uapC⁺* control and it shows the same background uptake rate of hypoxanthine as the triple-mutant recipient strain (Table II). On the other hand, the uric acid uptakes of strains carrying AC-2, -3 and -4 are higher than that of the negative control, while their growth phenotype is hardly distinguishable from it. If we subtract the background (40 pM/min/10⁷ conidiospores), the AC2-4 chimeras show a rate of uptake of uric acid of about half of the *uapC⁺* control. The disagreement between the uptake rates and the growth tests may only be apparent. To utilize uric acid as nitrogen source it is necessary that the intracellular steady state of uric acid be such that all the enzymes involved in purine utilization are induced. Uric acid itself is the physiological inducer of the *uapA*

Table I. Purine uptake rates of transformants carrying UapC/UapA (CA) chimeras

Strain	Purine uptake rates (pM/min/10 ⁷ conidia)		
	Substrate (20 µM)		
	8-C ¹⁴ -uric acid	8-C ¹⁴ -xanthine	G-H ³ -hypoxanthine
Wild-type (<i>uap⁺uapC⁺azg⁺</i>)	490±155	420±130	2200±250
ACZ (<i>uapA⁻uapC⁻azgA⁻</i>)	35±15	70±30	40±20
ACZ: <i>uapA</i> (pAN510)	405±100	410±135	55±5
ACZ: <i>uapC</i> (pAN910)	180±65	255±90	230±40
ACZ: CA-1	410±60	390±90	50±10
ACZ: CA-2	310±110	n.d.	50±15
ACZ: CA-3	380±100	n.d.	45±15
ACZ: CA-4	390±85	400±120	50±10
ACZ: CA-5	420±65	470±135	50±10
ACZ: CA-6	140±40	290±50	140±80
ACZ: CA-7	180±40	280±50	150±70

Only relevant genotypes are indicated (for complete genotypes see Materials and methods). Mean values and standard errors are shown (see Materials and methods).
n.d. = not determined.

Table II. Purine uptake rates for transformants carrying UapA/UapC (AC) chimeras

Strain	Purine uptake rates (pM/min/10 ⁷ conidia)	
	Substrate (20 μ M)	
	8-C ¹⁴ -uric acid	G-H ³ -hypoxanthine
Wild-type (<i>uap</i> ⁺ <i>uapC</i> ⁺ <i>azg</i> ⁺)	420 $\pm$ 90	1750 $\pm$ 500
ACZ		
(<i>uapA</i> ⁺ <i>uapC</i> ⁻ <i>azgA</i> ⁻)	40 $\pm$ 20	40 $\pm$ 10
ACZ: <i>uapA</i> (pAN510)	390 $\pm$ 125	60 $\pm$ 10
ACZ: <i>uapC</i> (pAN910)	150 $\pm$ 50	170 $\pm$ 40
ACZ: AC-2	80 $\pm$ 20	55 $\pm$ 15
ACZ: AC-3	80 $\pm$ 30	65 $\pm$ 15
ACZ: AC-4	100 $\pm$ 30	65 $\pm$ 10
ACZ: AC-5	180 $\pm$ 55	45 $\pm$ 10

For details see legend to Figure 1 and Materials and methods.

Table III. Purine uptake rates of transformants carrying an UapA/UapC/UapA (ACA) chimera

Strain	Purine uptake rates (pM/min/10 ⁷ conidia)	
	Substrate (20 μ M)	
	8-C ¹⁴ -uric acid	G-H ³ -hypoxanthine
Wild-type (<i>uap</i> ⁺ <i>uapC</i> ⁺ <i>azg</i> ⁺)	410 $\pm$ 70	2200 $\pm$ 440
ACZ		
(<i>uapA</i> ⁺ <i>uapC</i> ⁻ <i>azgA</i> ⁻)	40 $\pm$ 10	40 $\pm$ 20
ACZ: <i>uapA</i> (pAN510)	395 $\pm$ 110	55 $\pm$ 15
ACZ: <i>uapC</i> (pAN910)	150 $\pm$ 30	200 $\pm$ 70
ACZ: ACA-1	170 $\pm$ 40	30 $\pm$ 5
ACZ: ACA-(<i>inv</i>)	45 $\pm$ 15	50 $\pm$ 20

For details see legend to Figure 1 and Materials and methods.

and *uapC* genes (Gorfinkiel *et al.*, 1993; Diallynas *et al.*, 1995). Thus the apparent 'threshold effect' of uric acid uptake rates on uric acid utilization as nitrogen source may be due to inducer exclusion.

Construction and functional analysis of a chimeric UapA/UapC/UapA (ACA-1) transporter

Results with CA chimeras strongly suggest an important functional role of the region including amino acid residues 378–446 in UapA and 336–404 in UapC (Figures 1 and 2). To refine the analysis of this region, we replaced a 384 bp region of UapA with the corresponding region of chimeric gene CA-6 (Figure 4; see Materials and methods). This replacement led to chimera ACA-1, a protein like UapA, except that amino acid residues 357–446 of UapA were replaced with the corresponding amino acid residues of UapC (Figure 4). This region corresponds well to the putative functional region identified with the CA series of chimeras (378–446 in UapA, 336–404 in UapC). We also obtained, and used as a negative control, ACA-(*inv*), a chimera in which the same segment described above was inserted in a reverse orientation within the *uapA* open reading frame, thus resulting in early termination of translation. Constructions were confirmed by sequencing, introduced into *A.nidulans*, and transformants were obtained, purified and analysed as described previously.

Fig. 4. (A) Construction, (B) sequence and (C) topology of chimera ACA-1. (A) A region from chimeric gene CA-6 was amplified by a PCR using two oligonucleotides, P1 and P2, specific to *uapC* and *uapA* sequences, respectively (for details see Materials and methods). P1 bears a *Pst*I linker sequence at its 5' end. The amplified region of 460 bp includes a *uapA*-specific *Pst*I site. The PCR product was digested with *Pst*I and cloned by standard techniques into plasmid pAN510 replacing the homologous *Pst*I fragment. The resulting gene was named ACA-1. (B) Alignment of UapA and UapC amino acid sequences showing the UapA/UapC junctions in the ACA-1 chimeric protein. The amino acid sequence of the ACA-1 protein is shown in bold. (C) Predicted topology (see legend to Figure 2 for details) of ACA-1.

A single integration event at the *argB* locus was selected for further work. Table III shows that ACA-1 confers UapC-like uric acid transport but no hypoxanthine transport. These results support further the functional importance of amino acid residues 357–446 in UapA (315–404 in UapC) for uric acid (or xanthine) transport, but are surprising as far as it concerns hypoxanthine transport. This chimera shows no residual growth on hypoxanthine, which is consistent with the uptake determinations, but interestingly shows an extreme cryosensitivity on uric acid as sole nitrogen source (Figure 3).

Targeted mutagenesis of specific amino acid residues

The polar region between putative TMs 9 and 10 of UapA and UapC has 25 identical (33 similar) residues among a total length of 34 amino acids (Figure 5). This region lies within the sequences revealed by the studies with chimeric proteins to be of functional significance. Within this region, only residues E⁴¹² and R⁴¹⁴ in UapA are function-

Fig. 5. UapA point mutations constructed by *in vitro* oligonucleotide-directed mutagenesis (E412Q and R414E) or selected genetically (A404D, D404G and D404A). Top: UapA/UapC amino acid sequence alignment of the polar region including all point mutations characterized in this work. The boxed region represents the hydrophilic domain between the putative TMs 9 and 10. Middle and bottom: Location of the region shown in A in respect to the topology of UapA and of the minimal region involved in transport specificity, as defined by chimeric protein analysis.

ally different from the corresponding residues of UapC (Q³⁷⁰ and E³⁷², respectively). In these residues we replaced UapA with those found in UapC (Figure 4). The two single mutants E412Q and R414E, and the double mutant E412Q/R414E, were constructed on plasmid pAN510, confirmed by sequencing, and introduced as single copy in *A.nidulans* as previously described. Selected transformants were analysed for their ability to grow on various purines as nitrogen sources as well as for their capacity for ¹⁴C-uric acid or ³H-hypoxanthine accumulation. Strains carrying either single amino acid replacement are indistinguishable from the *uapA⁻uapC⁻azgA⁻* control in all growth tests. The strain carrying the double amino acid replacement has a novel phenotype. The double mutation results in inability to grow on uric acid but also in weak utilization of hypoxanthine, identical to that shown by strains transformed with an *uapC* wild-type gene. Uptake results (Table IV) are completely consistent with these growth tests. The single mutations E412Q or R414E result in total inability to transport both uric acid and hypoxanthine. The double mutation E412Q/R414E results in inability to transport uric acid, but in the acquisition of a low capacity to transport hypoxanthine (Table IV). These results strongly suggest that residues E412 and R414 are involved in UapA function and specificity.

Identification of an amino acid residue critical for uapA function

Mutation *uapA24* has been used as the standard loss of function allele and shown to result in total loss of UapA-dependent transport (Diallinas and Scazzocchio, 1989; Gorfinkel *et al.*, 1993; Diallinas *et al.*, 1995). *uapA24* was found to have a G-to-A transition resulting in an A⁴⁰⁴ to D⁴⁰⁴ change in the UapA protein (Figure 4; see Materials

Table IV. Purine uptake rates of strains carrying UapA point mutations

Strain	Purine uptake rates (pM/min/10 ⁷ conidia)	
	Substrate (20 μM)	
	8-C ¹⁴ -uric acid	G-H ³ -hypoxanthine
A ACZ		
(<i>uapA⁻uapC⁻azgA⁻</i>)	45 ± 10	40 ± 10
ACZ: <i>uapA</i> (pAN510)	410 ± 70	60 ± 20
ACZ: <i>uapC</i> (pAN910)	200 ± 60	170 ± 70
ACZ: pAN510–E412Q	50 ± 15	60 ± 10
ACZ: pAN510–R414E	55 ± 20	30 ± 10
ACZ: pAN510–E412Q/R414E	40 ± 20	110 ± 50
B <i>uapA⁺uapC⁻azgA⁻</i>		
<i>uapA24</i> (A404D)	520 ± 100	40 ± 20
<i>uapC⁻azgA⁻</i>	50 ± 10	60 ± 15
<i>uapA24-r1</i> (D404G)	550 ± 95	50 ± 15
<i>uapC⁻azgA⁻</i>		
<i>uapA24-r2</i> (D404A)	490 ± 120	55 ± 15
<i>uapC⁻azgA⁻</i>		

For details see legend to Figure 1 and Materials and methods.

and methods). Revertants of *uapA24* were isolated and shown to restore completely UapA-mediated transport (see Materials and methods). Genetic analysis of three of them show the reversion to be inseparable from *uapA24* in standard genetic crosses (data not shown). Sequencing of these revertant mutations showed that all three change the same codon mutated in *uapA24* (Figure 4). Two of them are back mutations restoring A⁴⁰⁴. The third one changes D⁴⁰⁴ to G⁴⁰⁴. A⁴⁰⁴ is located within the putative functional region identified by chimeric transporter analysis, eight and 10 amino acid residues upstream, respectively, from the putative functional amino acid residues identified by targeted mutagenesis (Figure 4). Uptake rates of strains carrying an *uapA24* mutation and the revertants described in the text are shown in Table IV.

Kinetic characterization of selected chimeras and mutants

Results with the CA series of chimeras suggest that specificity resides in the region defined by amino acid residues 336–404 in UapC and 378–446 in UapA. We wished to investigate the kinetic parameters of selected chimeras and mutations affecting this crucial region. Michaelis constant (*K_m*) and maximal velocity (*V*) values (all determined at 37°C) are shown in Table V and are strong agreement with the uptake data and the *in vivo* growth tests. It should be emphasized that these are apparent values, and that the *V_s* will also depend on the absolute amount of protein inserted in the membrane. The *K_m*s for uric acid are very similar for UapA and UapC (Table V), and thus it is not surprising that chimeras CA-5 and CA-6 have *K_m*s that are identical within the experimental error. The ACA-1 chimera has a significantly increased *K_m* compared with UapA and UapC, but a high *V*. The latter is absolutely consistent with its ability to utilize uric acid as a sole nitrogen source at 37°C (Figure 3; see below). The CA-6 chimera shows a *K_m* for hypoxanthine which is identical within the experimental error to

Table V. K_m and V values for uric acid and hypoxanthine uptake of transformants expressing UapA, UapC, chimeric transporters CA-5, CA-6 and ACA-1, and the E412Q/R414E UapA double mutant

Strain	K_m (for uric acid)	V	K_m (for hypoxanthine)	V
ACZ: <i>uapA</i> (pAN510)	6.5±1	610±70	N.T.	N.T.
ACZ: <i>uapC</i> (pAN910)	7.5±2	240±80	25±5	270±60
ACZ: CA-5	7.0±1	550±80	N.T.	N.T.
ACZ: CA-6	8.0±1	190±85	28±3	170±60
ACZ: ACA-1	15.0±2	340±90	N.T.	N.T.
ACZ: pAN510-E412Q/ R414E	N.T.	N.T.	148±9	280±30

K_m and V values were calculated by double reciprocal Lineweaver-Burk plots and represent average values for three independent experiments. The substrate range chosen was optimized for the determination of K_m s. For experimental details see Materials and methods. K_m in μ M, V in pM/min/10⁷ conidia.

N.T. = no transport detected even at external substrate concentrations of 300 μ M.

that of the UapC permease. The E412Q/R414E mutation of *uapA* results in K_m for hypoxanthine being substantially higher than that of UapC. This is consistent with the results with the ACA-1 chimera which indicated that residues other than the ones swapped in this chimera are important for hypoxanthine recognition and/or transport. It is satisfying that the V for the UapA(E412Q/R414E) mutant is of the same order of that shown by the UapC wild-type protein. This fits very nicely with the *in vivo* tests, which are done at concentrations of 600 μ M for the different purines, far above the K_m values, and the strain carrying the E412Q/R414E mutation shows a level of growth on hypoxanthine which is indistinguishable from that shown by a strain carrying the *uapC*⁺ plasmid.

Evidence that the region identified by chimeric transporters includes a hypoxanthine binding site

Results with the CA series of chimeras suggest that the hypoxanthine binding site is the region defined by amino acid residues 338–404. However, results with the AC series and with the ACA-1 chimera suggest that this region is not sufficient to confer the ability to transport hypoxanthine (see Discussion). We tested the presence of a hypoxanthine-binding site in the region defined above by uptake competition experiments. We measured, in selected chimeras, uric acid uptake in the presence and absence of excess cold hypoxanthine. Results summarized in Table VI showed that ¹⁴C-uric acid transport by UapC, or by chimeric transporters containing UapC-derived amino acid residues in the region 1–404 (CA-6), 279–580 (AC-4), or 315–404 (ACA-1), is significantly reduced (up to 96% in ACA-1) in the presence of excess hypoxanthine. On the other hand, ¹⁴C-uric acid transport by UapA or by chimeric transporters CA-5 or AC-5, which contain UapC-derived amino acid residues in the region 1–335 or 405–580, respectively, is little or not affected by the simultaneous presence of hypoxanthine. These results strongly suggest that amino acid residues 336–404 of UapC, present in CA-6, ACA-1 and AC-4, contain a hypoxanthine-binding site. Hypoxanthine concentrations resulting in 50% uric acid uptake inhibition were found to be very similar to the previously determined hypoxanthine K_m values for UapC, CA-6 and ACA-1 (Table VI).

***In silico* identification of homologues of purine transporters**

We have previously defined a class of transporters which show clear sequence similarity and extremely similar predicted topology in a multiple alignment. This family included, together with UapA and UapC of *A.nidulans* and known nucleobase transporters from *E.coli*, *Bacillus subtilis* and *Bacillus caldolyticus*, a number of bacterial sequences of unidentified function present in the databases.

Mouse (Yspl1) and maize (Lpe1) homologues have since been described (Guimarães *et al.*, 1995; Schultes *et al.*, 1996). We have now identified in the databases possible homologues from a number of other organisms. A search conducted while this work was being revised revealed a total of 36 membrane proteins belonging to this class. These possible homologues appear as first strikes in searches in the databases of different organisms when the whole *uapC* peptide sequence was used as an *in silico* probe. These homologues are found in quite diverse bacteria, including *Thermotoga maritima* and *Deinococcus radiodurans*, which are not particularly close to *E.coli* and *Bacillus* spp. in the phylogenetic scale (Figure 6). We have failed to identify possible homologues in the available sequences of Archea. Putative homologues are present in a number of dicotyledonous plants (Figure 6). We have also identified several possible metazoan homologues. These include *C.elegans*, mouse, an incomplete cDNA coding for 380 residues from human bone marrow and a short EST from ovarian cancer, which nevertheless contains the critical functional domain defined in this article. One of the two mouse homologues had previously been identified as a cDNA coding for a putative nucleobase transporter and expressed in the haematopoiesis occurring in the yolk sac (Guimarães *et al.*, 1995). The human bone marrow homologue shows 24 and 26% identity with UapC and UapA, respectively, and 32 and 34% identity, respectively, with the mouse homologues. The identities of the mouse homologues are 35% between themselves and 22–24% with the *A.nidulans* proteins. The two *C.elegans* homologues show an identity of 57% between themselves, 22–25% with the *A.nidulans* nucleobase transporters, 25–28% with the mouse homologues and 35–36% with the human bone marrow homologue. A possible homologue of *Drosophila melanogaster* (AA39031) has also been identified but is not included in Figure 6 (see below) as the sequenced region does not extend to the critical region defined in this article.

The conservation of sequence is particularly striking in the region defined as essential for specificity by the chimeric and mutational analysis described above (Figure 6). The sequence identity and similarity of the metazoan homologues is higher with the known purine than with the known pyrimidine transporters, which may be an indication of the specificity of these proteins. A short, highly conserved region with the consensus sequence (F/Y/S)X(Q/E/P)NXGXXXXT(K/R/G) (the bold Xs indicate residues that, while not conserved, are always aliphatic hydrophobic amino acids) is present in the critical domain defined in this article. In Figure 6, the conservation of this region is shown for representative possible nucleobase transporter homologues identified in the database. This sequence is located in a region of complete identity between UapA and UapC, within the region defined by

Table VI. Competition of uric acid uptake by the simultaneous presence of hypoxanthine in transformants carrying chimeric transporters

Strain	Purine uptake rates (pM/min/10 ⁷ conidia)		Hypoxanthine concentration resulting in 50% inhibition uptake of 8 μ M uric acid (μ M)
	20 μ M 8-C ¹⁴ -uric acid	20 μ M 8-C ¹⁴ -uric acid + 200 μ M hypoxanthine	
ACZ: <i>uapA</i> (pAN510)	345 $\pm$ 120	410 $\pm$ 40	N.C.
ACZ: <i>uapC</i> (pAN910)	170 $\pm$ 80	30 $\pm$ 20	29 $\pm$ 5
ACZ: CA-5	380 $\pm$ 110	260 $\pm$ 85	N.C.
ACZ: CA-6	165 $\pm$ 15	60 $\pm$ 25	23 $\pm$ 6
ACZ: AC-4	90 $\pm$ 35	10 $\pm$ 5	n.d.
ACZ: AC-5	140 $\pm$ 55	95 $\pm$ 30	n.d.
ACZ: ACA-(<i>inv</i>)	20 $\pm$ 20	20 $\pm$ 10	n.d.
ACZ: ACA-I	240 $\pm$ 60	10 $\pm$ 0	4 $\pm$ 1

For experimental details see legend to Figure 1 and Materials and methods.

N.C. = no competition detected even at 200 μ M hypoxanthine.

n.d. = not determined.

Fig. 6. An amino acid 'signature' sequence (F/Y/S)X(Q/E/P)-NXGXXXXXT(K/R/G) present in all members of the nucleobase transporter family (representative members shown). A bold X represents aliphatic hydrophobic amino acids. UapA and UapC are described in this work. PbuX is a xanthine transporter of *B.subtilis*. PyrP1 and PyrP2 are pyrimidine transporters of *B.subtilis* and *B.caldolyticus*, respectively. UraA is a uracil transporter of *E.coli*. Lpe1 is a maize homologue which was shown to be essential for chloroplast development (Schultes et al., 1996). YsP1 is a mouse protein, preferentially expressed in the yolk sac (Guimarães et al., 1995). Transport activity has not been investigated for these last two proteins. All other sequences derive from databases and show overall similarity with the proteins known to be nucleobase transporters. The *S.cerevisiae* FCY2 protein is a purine-cytosine transporter which does not belong to this family of transporters, but may contain a similar functional motif. Accession numbers are indicated for all proteins.

the CA-5 and CA-6 chimeras as important for UapA and UapC function and specificity, immediately downstream of the tenth putative TM (TM10).

Discussion

Chimeric protein and mutant analysis leads to a central conclusion. A region including TM9 and TM10 and hydrophilic segments 9, 10 and 11 (corresponding to amino acid residues 378–446 in UapA, or 336–404 in UapC) determines uptake specificity. The corresponding UapC region carries a hypoxanthine binding domain as shown also by competition experiments. Among the 69 amino acids included in the specificity region defined by the CA series of chimeras, we find 32 differences between UapA and UapC. Twenty-nine of these amino acid differences correspond to conserved amino acid changes. Two residues in this region, R414 and E412, are indeed critical for the function and specificity of UapA. These residues are good candidates to form part of a purine binding site. A third neighboring amino acid residue, A404, is also important for function, but probably not for specificity *vis-à-vis* purines. It is striking that in all 36 transporters of this class identified in the databases, the residue at this position is either an alanine or a glycine, in agreement with the fact that in UapA, A404 can be functionally replaced by G404.

In the previously proposed 14 α -helical model (Diallinas et al., 1995), the amino acid residues 404, 412 and 414 of UapA, which were shown to be important for function and specificity, would be located in the segment joining TM9 and TM10, that is, exposed to the external environment. In the same model, the putative functional and conserved motif (F/Y/S)X(Q/E/P)NXGXXXXXT(K/R/G) would be located immediately downstream from TM10, facing the inside of the cell. A specific region of FCY2, (Bloch et al., 1992; Ferreira et al., 1996; the purine-cytosine permease of *S.cerevisiae* belonging to a different family of membrane proteins) which has been found to be involved in the recognition of its substrates, is in a similar topological position and shows some sequence similarity with the motif described above (Figure 6). While this work was being revised, Wang and Giacomini (1997) published results that suggest very strongly that a discrete region within the sequences comprised in TM8–9 deter-

mines the purine versus pyrimidine specificity of the 14 TM rat nucleoside transporters. In the twelve TM domain human glucose transporters (GLUT2, 3, 4), it has been established that the region bracketed by TM7–10 is most critical for substrate recognition, while other N-terminal domains may contribute to substrate specificity in GLUT1 and GLUT2 (Arbuckle *et al.*, 1996; Wandel *et al.*, 1996; Noel and Newgard, 1997).

The results with the AC and ACA chimeras add an additional twist to the apparently simple, linear pattern of substrate specificity discussed above. The phenotypes of these chimeras strongly suggest that the UapA N-terminus is incompatible with a UapC-like protein. Even AC-2, which contains mostly UapC sequences (Figure 2), shows a strong mutant phenotype. Only when the crucial putative tenth hydrophilic segment is included (in AC-5) is a clearly functional, albeit cold-sensitive, protein obtained. It should be noted that the hydrophilic N-terminus of UapA is much longer and more acidic than the corresponding region of UapC and all other transporters of this group. The properties of the ACA-1 chimera show that the 336–404 sequence does not generate an incompatible phenotype in a UapA context. If this was so, the chimera would have a phenotype identical with AC-2, -3 and -4, which it has not. The phenotypic similarity *vis-à-vis* uric acid uptake of ACA-1 and AC-5, including their cryosensitivity, is perplexing since their structure is very different; AC-5 is mostly UapA with an UapC C-terminus, while ACA-1 is an UapA protein with a UapC specificity region. Most notably, AC-5 contains the UapA specificity region. It should also be noted that the ACA-1, but not the AC-5 chimera contains a hypoxanthine-binding site. The properties of the UapA E412Q/R414E double mutant are compatible with the explanation proposed above, as only two residues have been changed to the corresponding UapC residues and thus this protein would not contain 'incompatible' UapA and UapC sequences.

The non-equivalence of the results with the CA series on the one hand and AC and ACA-1 chimeras on the other underlines the biased interpretation which may result from studying chimeras constructed by only one series of linear permutations (Caron, 1993). Other studies with chimeric proteins have shown similar apparent contradictions. The function of a specific chimera may be lost or impaired by protein misfolding, functional impairment or improper plasma membrane targeting (Caron, 1993; Buck and Amara, 1994; Zhang *et al.*, 1995; Wang and Giacomini, 1997). The data presented in this article establish that the region defined by TM9–10 is the 'minimal domain' required for substrate specificity of the UapA and UapC proteins, but also that other regions are involved in determining this specificity. In particular, the comparison of the properties of the CA series of chimeras with those of the ACA-1 chimera establish that the hypoxanthine-binding site carried by the 'C' region of this protein is necessary but not sufficient for hypoxanthine uptake.

All chimeras of the CA series lead to either a typical *uapA*⁺ or *uapC*⁺ phenotype. This makes us confident, in the absence of immunological detection of the purine transporters in mycelia, that the proteins are expressed and correctly inserted in the membrane. The high *V*_s obtained with the ACA-1 chimera and the UapA(E412Q/R414E) mutant are also consistent with an absolute quant-

ity of protein inserted into the membrane of the same order of that present in strains expressing wild-type *uapA* and *uapC* genes. In contrast, the most straightforward explanation of the phenotype of the AC-2, -3 and -4 chimeras is that folding and/or membrane insertion are defective. The cryosensitivity of AC-5 and ACA-1 suggests strongly that proper folding of the proteins is impaired, albeit less than in AC-2, -3 and -4. Only a series of antibodies against different epitopes of both proteins can lend support to the above proposal. We have obtained antibodies against a series of epitopes of UapA and UapC. These were raised both against fusion proteins synthesized in *E.coli* and synthetic peptides. While these antibodies recognize the cognate epitopes at very high titers, we have been unable to detect UapA and UapC whole proteins in Western blots of mycelial extracts or membrane fractions. Even in a strain carrying a *uapA100* promoter mutation [resulting in an increase of about eight times of *uapA* message and uric acid uptake (Arst and Scazzocchio, 1975; Gorfinkiel *et al.*, 1993)] the UapA protein is undetectable (J.Valdez, G.Diallinas, C.Scazzocchio and A.Rosa, unpublished results). This is probably due to the extremely low expression levels of many transporters. In fact, only drastic overexpression of the yeast uracil, FUR4, and purine-cytosine, FCY2 transporters (about 40 times for FCY2) results in levels detectable in Western blots (Volland *et al.*, 1994; Rodriguez *et al.*, 1995; Galan and Haguenaer-Tsapis, 1997).

We have referred to the general importance of nucleobase transport in animal, and more specifically human cells. In this article we describe for the first time a functional domain carrying the specificity determinants in a nucleobase transporter. The domain, defined by chimeric protein and mutational analysis as essential for specificity, has assisted the *in silico* analysis in the identification of bacterial, plant and metazoan homologues of purine transporters, including the detection of an universally conserved 'signature' sequence. The similarity in amino acid sequence and topology of purine transporters from bacteria to man could also establish *A.nidulans* as a unique genetic and molecular system for studying heterologous purine transporters.

Materials and methods

Media and growth conditions for *A.nidulans*

These have been described previously (Cove, 1966; Scazzocchio *et al.*, 1982). Supplements were added when necessary.

Strains

The following otherwise isogenic *A.nidulans* strains were used: *biA1*; *pabaA1*; *yA2 pabaA1*; *uapA24 pabaA1*; *uapA24 azgA4 pabaA1*; *azgA4 pabaA1*; *uapC201 uapC401 pabaA1*; *uapC201 uapC401 uapA24 pabaA1*; *uapC201 uapC401 azgA4 pabaA1*; *uapC201 uapC401 uapA24 azgA4 pabaA1*; *yA2 argB2 uapC201 uapC401 uapA24 azgA4*. *biA1*, *pabaA1* and *argB2* indicate auxotrophies for biotin, *p*-aminobenzoic acid and arginine, respectively. *yA2* results in yellow conidia. None of these markers affects the regulation of gene products involved in purine catabolism. *uapA24*, *azgA4* and *uapC401* are loss-of-function mutations in the corresponding genes. *uapC201* is a promoter mutation (Diallinas *et al.*, 1995). Double and triple mutants were constructed by standard *A.nidulans* genetic techniques.

DNA manipulations and plasmid construction

Plasmid isolation from *E.coli* strains and DNA manipulations were performed as described by Sambrook *et al.* (1989). Total genomic DNA

isolation from *A.nidulans* strains has been previously described by Lockington *et al.* (1985). Chimeric genes or *uapA* alleles were entirely sequenced by the dideoxy/T7 polymerase method using a polymerase chain reaction (PCR)-sequencing kit as instructed by the supplier (United States Biochemicals, Cleveland, OH). A series of oligonucleotides specific for both strands of the *uapA* and *uapC* genes [previously described in Gorfinkiel *et al.* (1993) and Diallinas *et al.* (1995)], and the T3 and T7 primers (Pharmacia) were used for sequencing. Control plasmids pAN910 and pAN510, including the *uapC* and *uapA* genes, respectively, and genes for selection in *E.coli* (*bla*) and *A.nidulans* (*argB*), were constructed from previously described plasmids pAN503 (Gorfinkiel *et al.*, 1993), pAN903 (Diallinas *et al.*, 1995) and pJL16 (Johnstone *et al.*, 1985). pAN510, a 2.3 kb *Sall*–*Sall* restriction fragment containing the *argB* gene was isolated, after partial digestion, from pJL16 and cloned into the unique *Sall* site of pAN503. pAN910, a *BglII*–*BamHI* 2.7 kb fragment containing the *uapC* gene was cloned into the *BamHI* site of pJL16. pAN590, used for the isolation of CA chimeric genes, was constructed by cloning a *BglII*–*BglII* 4.4 kb fragment, containing *uapA* and part of *argB*, from pAN510 into the unique *BamHI* site of pAN910. pAN590, used for the isolation of AC chimeric genes, was constructed in two steps, as follows; a 3.4 kb *Sall*–*Sall* restriction fragment from pAN903 containing *uapC* was cloned into the unique *Sall* site, downstream from the *uapA* gene, of plasmid pAN503. The resulting plasmid was named pAN5090. Then, a 3.3 kb *XbaI* restriction fragment, containing the *argB* gene, from pJL16 was inserted into a unique *SpeI* site of pAN5090. Plasmids were introduced to *E.coli* or *A.nidulans* by standard transformation techniques (Diallinas and Scazzocchio, 1989; Sambrook *et al.*, 1989).

In vivo construction of chimeric CA and AC genes

Chimeric *uapC/uapA* (CA) or *uapA/uapC* (AC) genes were constructed *in vivo* by homologous recombination of plasmids pAN950 or pAN590, respectively, in *E.coli*. The technique used was basically as described by Moore and Blakely (1994). Isolation of chimeric CA genes was as follows: 10 µg of pAN950 were digested with *SnaBI*, a restriction enzyme which has a unique target site located 220 bp upstream from the translation end of *uapC*. Thus, *SnaBI* digestion results in linear pAN950 molecules including the *uapA* and *uapC* genes at their two edges. Linearized pAN950 was used to transform *E.coli* DH5α. One hundred and four ampicillin resistant transformants were selected and analysed with restriction digests; 88 were identical to the original plasmid pAN950; 16 were the result of homologous recombination between *uapC* and *uapA* sequences leading to chimeric CA genes. Complete sequence analysis of 12 chimeric CA genes identified seven different unique recombination events which have occurred at various sites along the *uapC* and *uapA* sequences (CA-1, -2, -3, -4, -5, -6 and -7). Isolation of chimeric AC genes was as follows: 10 µg of pAN590 were digested with *NruI*, a restriction enzyme which has a unique target site located downstream from *uapA*. Linearized pAN590 was used to transform *E.coli* DH5α. Ninety-two ampicillin-resistant transformants were selected and analysed with restriction digests; 80 were identical to the original plasmid pAN590; 12 were the result of homologous recombination between *uapA* and *uapC* sequences leading to chimeric AC genes. Sequence analysis of all of them led to the identification of five different AC genes.

Construction of a chimeric ACA gene

ACA-1, a chimeric gene in which a region of 282 bp in *uapA* (corresponding to amino acid residues 352–446) was replaced by the homologous region of *uapC*, was constructed as follows: the region encoding amino acid residues 309–473 of chimeric gene CA-6 was amplified by 30 cycles (94°C 5 min; 94°C 45 s; 58°C 1 min; 72°C 2 min) of the PCR using the oligonucleotides P1 (5'-AACTGCA-GCCTGTGGCTACTTCGACCGC-3') and P2 (5'-CCTTGGCCACGAT-CGCC-3'). The amplified 500 bp PCR product contained two *PstI* sites; one at its 5' end inserted as a linker attached to oligonucleotide P1 and a second located within the amplified sequences. Upon *PstI* digestion, a 402 bp *PstI*–*PstI* fragment was isolated and used to replace the *PstI*–*PstI* region from the *uapA* gene in plasmid pAN510. Sequence analysis of a number of recombinant plasmids isolated in *E.coli* permitted the identification of constructs in which the *PstI*–*PstI* insert was cloned in opposite orientations. A construct carrying the insert in the correct orientation which re-establishes the open reading frame in the chimeric *uapA/uapC/uapA* gene was named ACA-1. This construct encodes a chimeric protein in which amino acid residues 352–446 of UapA have been replaced by the homologous amino acid residues of UapC (310–404). A construct carrying the insert in an inverted orientation, named

ACA-(*inv*), was also isolated and used in subsequent experiments as a control plasmid which carries a disrupted *uapA* gene.

Construction of specific uapA mutations

Specific *uapA* mutations were constructed *in vitro* by oligonucleotide-directed targeted mutagenesis using a Muta-gene M13/*dut*,*ung*/T4 polymerase bacterial Kit according to the manufacturer's instructions (Bio-Rad). Mutations were initially constructed into a 460 bp *PstI*–*PstI* restriction fragment from *uapA* cloned in pBluescriptII (KS+). Constructs were confirmed by sequencing the entire *PstI*–*PstI* restriction fragment. The mutated fragment was subsequently used to replace the corresponding *PstI*–*PstI* restriction fragment in pAN510. The oligonucleotides used to construct single mutations E412Q and R414E, as well as the double mutation E412Q/R414E were the following:
EQ: 5'-GTCTCGCGTCTCCAAGTCCGCGGCGGC-3',
RE: 5'-CGTCTCGAAGTCGAGGGCGGCACATTCG-3' and
EQRE: 5'-GTCTCGCGTCTCCAAGTCGAGGGCGGCACATTCG-3'.

Characterization of uapA24 and uapA24 revertants

uapA24 has been described previously (Diallinas and Scazzocchio, 1989; Gorfinkiel *et al.*, 1993; Diallinas *et al.*, 1995). Characterization of *uapA24* was carried out by amplification and direct sequencing of a 2.1 kb genomic DNA fragment including the *uapA24* allele. Amplification was carried out by a standard PCR (94°C 5 min; 94°C 45 s; 58°C 1 min; 72°C 2 min for 30 cycles; 72°C 15 min) using as primers two 'external' *uapA*-specific oligonucleotides, UAPA1 (5'-GACCCATCTTCCACCA-TG-3') and UAPA8 (5'-CGGTGGACATGTCACTCG-3'). Revertants of *uapA24* were isolated after UV mutagenesis (lethality rate 92%) of 10⁸ conidiospores of a *uapC201 uapC401 uapA24 azgA4 pabaI* mutant strain and selection for growth on uric acid as sole nitrogen source at 25°C. Selection at 25°C reduces residual background growth due to minor, genetically non-defined purine transporters. Six mutants able to grow on uric acid were selected. All showed a *uapA*⁺ growth phenotype. Upon back-cross to a *uapA*⁺ strain, three of them were shown to include mutations genetically linked to *uapA*. All three were identified by PCR amplification and direct sequencing as described above for *uapA24*.

Purine transport assays

8-¹⁴C-uric acid, 8-¹⁴C-xanthine or G-³H-hypoxanthine uptake was assayed in conidiospores as described previously (Diallinas *et al.*, 1995; Tazebay *et al.*, 1995). All transport assays were carried out at least in triplicate. Means and standard errors are shown for all determinations. Uniformly labeled 8-¹⁴C-uric acid (specific activity 1.94 GBq/mmol), 8-¹⁴C-xanthine (2.1 Gbq/mmol) and G-³H-hypoxanthine (specific activity 244 GBq/mmol) were obtained from Isotopchim, Ganagobie-Peyruis, France and Amersham International, England.

Determination of kinetic parameters

The apparent *K_m* and *V* values for 8-¹⁴C-uric acid or G-³H-hypoxanthine were determined from double reciprocal plots of the initial uptake rate against substrate concentration. Initial velocities were corrected by subtracting background uptake values, evident in the triple *uapA uapC azgA* mutant (Tables I, II, III and V), which may be due to as yet genetically undefined minor purine transporters present in all strains used in uptake assays. For *K_m* and *V* determination, an optimized substrate concentration range was chosen. For uric acid uptake this was 1, 2, 5, 10, 20, 50 or 100 µM, and for hypoxanthine uptake this was 5, 10, 20, 40, 80, 150 or 300 µM. To calculate the concentration of hypoxanthine which results in 50% of 8-¹⁴C-uric acid uptake inhibition we used a constant uric acid concentration close to the UapA and UapC *K_m* (8 µM), and a hypoxanthine concentration range of 10, 20, 50, 100 and 200 µM. All transport assays were carried out in triplicate. The errors given in tables are standard errors of the mean value.

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Note added in proof

There is a mistake in the *uapA* sequence in Figures 1 and 4. Residue 430 should be an I, not a T. This error is due to a mis-reading of an ATC (I) codon as an ACT (T). This mistake has been corrected in the database. Note that this increases the identity of *uapA* and *uapC* in the critical specificity region.