A Gene Coding for the Uric Acid-Xanthine Permease of Aspergillus nidulans: Inactivational Cloning, Characterization, and Sequence of a cis-Acting Mutation

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ABSTRACT

In Aspergillus nidulans, integration of transforming sequences can proceed through recombination with homologous sequences or at heterologous sites in the genome. In a strain with a large deletion in the gene coding for acetamidase (amdS), a plasmid carrying this gene integrates into and inactivates uapA, the putative structural gene for uric acid-xanthine permease, with a frequency of 0.3%. The integration event occurs 3' to the open reading frame of amdS. A 10-nucleotide sequence which occurs in this region is also found within the open reading frame of uapA. We have taken advantage of this integration event to clone the permease gene and to characterize a cis-acting mutation, uap-100, as a duplication of 139 bp located in the upstream region of uapA. Northern and dot blot analyses confirmed earlier results measuring the uptake of uric acid: the transcription of the uapA gene is inducible and the uap-100 mutation results in a bypass of the need for induction while having an 8-fold up-promoter effect under inducing conditions.

IN Aspergillus nidulans, as in other filamentous fungi, integration of transforming sequences can occur at both homologous and heterologous locations. In particular, heterologous integration events of plasmids carrying the amdS gene, coding for acetamidase, have been previously described (Tilburn et al. 1983; Wernars et al. 1985). Tilburn (1988) has shown, using amdS deletions, that when the homology between the transforming amdS plasmid and the resident chromosomal DNA was about 1.5 kbp, only 1 out of 19 transformants analyzed contained an integration in the residual chromosomal amdS sequences. If heterologous integration occurs at random, it may be possible to use it as a means of cloning genes by inactivation in every case where a selection for loss of function is available.

To investigate the randomness of integration events we devised the following system. We used a strain with a large deletion in the acetamidase gene, amdS368 (Hynes 1979), and a plasmid containing the entire amdS structural gene plus essential control sequences but shorter than the sequences deleted in amdS368 (Hynes et al. 1988). To detect integration events at a number of different genes we used a technique described by Alderson and Sczzocchio (1967). In the presence of the xanthine analog 2-thioxanthine, wild-type strains of A. nidulans give yellow rather than green conidia. This effect requires the uptake of the analog and its oxidation to 2-thiouric acid which presumably acts by chelating the copper of laccase (polyphenol oxidase) which is involved in the conversion of yellow to green pigment (Sczzocchio 1966; Alderson and Sczzocchio 1968). Mutations at any locus necessary for this process will result in resistance to 2-thioxanthine, i.e., in the production of green conidia even in the presence of 2-thioxanthine. Loci at which mutations result in 2-thioxanthine resistance, and the rationale for the mutant phenotype, are shown in Table 1. Very easy growth and/or complementation tests permit the assignment of any given mutation, or integration event, to a given gene (Darlington and Sczzocchio 1967; Alderson and Sczzocchio 1968). In this article we show that a plasmid carrying amdS sequences integrates preferentially at the uapA gene encoding the uric acid-xanthine permease. We made use of one such event to obtain genomic clones containing this gene.

The expression of uapA requires specific induction by uric acid or its thio-analogs and the presence of the narrow-domain uay and the wide-domain areA regulatory gene products. The expression of uapA is impaired specifically in strains carrying the areA102 allele. We have previously described a cis-dominant and trans-recessive mutation, uap-100, which maps adjacent to uapA and has a number of effects on the regulation of the permease gene (Arst and Sczzocchio 1975; Sczzocchio and Arst 1978). This mutation results in a 2.5-fold up-promoter effect, a bypass of the need for induction still leaving the mutant dependent on the presence of an active uay product (Sczzocchio and Arst 1978; Sczzocchio, Sdrin and Ong 1982), and a bypass of the areA102 pheno-
type (ARST and COVE 1973) still leaving the mutant dependent on the presence of an active areA product (ARST and SCAZZOCCHIO 1975). In this article, we show that uap-100 is a 139-bp duplication which increases uapA transcription and that the amds integration events occur downstream from the uap-100 duplication site, within the uapA open reading frame. Also, we present results giving interesting insights on the effect of uap-100 on the expression of the uapA gene.

**MATERIALS AND METHODS**

**Strains:** The following *Aspergillus nidulans* strains were used: amds368 amdl18 amdA7 niaA4 biaA1; amds368 amdl18 amdA7 niaA4 uapA24 bia1 pantOB100; fua1 areA102 pyroA4; uap-100 bia1; bia1; ya2 amds320 amdl18 amdA7 niaA4 pantOB100 bia1; and ya2 uapA24 pantOB190.

*amds368* is a 6.0-kbp deletion of the *amds* gene coding for acetamidase and *amds320* is a 2.8-kbp deletion of the same gene (HYNES 1979; HYNES et al. 1983). *uapA24* is a mutation in the structural gene for the uric acid-xanthine permease (ARST and SCAZZOCCHIO 1975). *uap-100* is a cis-acting mutation which has been discussed in earlier sections. *areA102* is a mutation in the wide domain regulatory gene *areA* involved in nitrogen metabolite repression (ARST and COVE 1973). It results in enhanced expression of a number of genes involved in the utilization of nitrogen sources, but abolishes the expression of the gene for uric acid-xanthine permease and diminishes the expression of the gene coding for formamidase (HYNES 1979; ARST and COVE 1973; ARST and SCAZZOCCHIO 1975). *amdA7* and *amdl18* are mutations resulting in elevated levels of *amds* expression and have been described in detail by HYNES (1978a, b, 1979) and HYNES et al. (1988). *niaA4* is a mutation in the structural gene for nitrite reductase (PATEMAN and COVE 1967). *ya2* and *fua1* result in yellow and fawn conidiospores, respectively. *bia1*, *pantOB100* and *pyroA4* indicate auxotrophies for biotin, pantothenic acid and pyridoxine, respectively.

**Escherichia coli** K12 strain DH1 (LOW 1968) was used for routine plasmid preparations. *E. coli* strains JM101 and JM109 (YANISCH-PERRON, VIEIRA and MESSING 1985) were used for the propagation of *M. luteus* phases. *E. coli* Q359 (KARN et al. 1980) was used for the propagation of λ EMBL4 recombinant phases.

**Media and growth conditions:** The standard media and growth conditions for *A. nidulans* were used as described by COVE (1966). Mycelia from which protoplasts were prepared were grown as described by TILBURN et al. (1983). Mycelia for DNA preparations were grown in appropriately supplemented minimal media for 18 hours at 25°, shaken at 120 rpm. Mycelia for RNA preparations were grown for 21 hr at 120 rpm on appropriately supplemented minimal media, plus 1% glucose as carbon source and 5 mM urea as nitrogen source (noninducing conditions; NI) or on the same media to which 27 μM 2-thiouric acid was added after 16 hr and growth allowed to continue for a further 5 hr (inducing conditions; I).

**Isolation of nucleic acids:** *A. nidulans* DNA and RNA preparations were as previously described (TILBURN et al. 1983; LOCKINGTON et al. 1985). Plasmid preparations were as described by MANIATIS, FRITSCH and SAMBROOK (1982). poly(A)* RNA was isolated as described by WERNER, CHEMLA and HERZBERG (1984).

**Transformation:** *A. nidulans* protoplasts were prepared as described by TILBURN et al. (1983). In each transformation experiment, protoplasts were aliquoted. pAMD21 was used as the transforming plasmid in three experiments. Experiment 1 involved 12, experiment 2, 15 and experiment 3, 30 aliquots, respectively. DNA (25, 10 and 10 μg in experiments 1, 2, and 3, respectively) and polyethylene glycol were individually added to such aliquots and the procedure was continued as detailed in TILBURN et al. (1983). Each aliquot was used to inoculate a tube containing 4 ml of soft agar which was used to overlay a single Petri dish. Controls without DNA and regeneration controls were performed in an analogous manner. *E. coli* transformation was performed according to HANAHAN (1983).

**Genetic techniques:** *Aspergillus* genetic techniques (crossing, selfing, etc.) were performed as described by PONTECORVO et al. (1953).

**DNA manipulations and sequencing:** Restriction digests of *Aspergillus* or plasmid DNA were performed as described by MANIATIS, FRITSCH and SAMBROOK (1982). Ligation of plasmid DNA was performed as described by MURRAY (1986). DNA sequences were determined with the dideoxynucleotide chain termination method (SANGER, NICKLEN and COULSON 1977) using M13mp18 and M13mp19 single-stranded templates as described by MESSING (1983). Single- and double-stranded DNA of *M. luteus* subclones was prepared as described by the *Amerasheii* M13 Cloning and Sequencing Handbook (1983).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Nitrogen source and purine analogs</th>
<th>Ammonium d-tartrate</th>
<th>2-thiouric acid</th>
<th>Hypoxanthine</th>
<th>Uric acid</th>
<th>Sodium nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class of mutant</td>
<td>Ammonium d-tartrate</td>
<td>2-thiouric acid</td>
<td>Hypoxanthine</td>
<td>Uric acid</td>
<td>Sodium nitrate</td>
</tr>
<tr>
<td>hxA, hxB</td>
<td>+</td>
<td>s</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>uapA</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cnx ABC,E,F,G,H</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>uapA</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* indicates normal growth; - indicates lack of growth; ± indicates the leaky growth typical of uapA mutants; r and s indicate resistance and sensitivity to 2-thioxanthine and 2-thiouric acid, respectively. Allopurinol specifically inhibits the utilization of hypoxanthine as a nitrogen source but is taken up through the uric acid-xanthine uptake system. Mutations in the hxA and hxB genes, as well as in the cnx genes, can be easily distinguished from each other by complementation tests. For the rationale of all these tests see DARLINGTON and SCAZZOCCHIO (1967), ALDERSON and SCAZZOCCHIO (1968) and SCAZZOCCHIO and ARST (1978).
[$^{35}$S]dATP labeled DNA molecules of different lengths were separated on 6% acrylamide/8 M urea gels. All possible Sau3AI and NheI restriction fragments of the wild-type and \textit{uap-100} BglII-PstI region (see Figure 5) were cloned into M13 vectors and sequenced in both directions.

**Transfers and Hybridizations:** Probes were labeled by nick-translation as described in the \textit{Amersham Bulletin} (1987). Southern and Northern transfers, as well as dot blots, were as described by Maniatis, Fritsch and Sam-Brook (1982). Nitrocellulose was baked for 2 hr at 80° under vacuum. Prehybridization and hybridization were performed as described by Boyer (1986). To monitor poly(A)* RNA concentrations we used an actin-specific probe (plasmid pSF5). Densitometry readings enabled us to compare the levels of \textit{uapA} expression under non-induced and induced conditions in a wild-type and a \textit{uap-100} background.

**Plasmids and gene libraries:** Plasmid pAMD21 was a gift from M. Hynes. It carries an intact \textit{A. nidulans} \textit{amdS} gene and essential control sequences contained in an EcoRI-SmaI fragment cloned into pUC9 (Hynes et al. 1988). Plasmid pSF5 was a gift from R. Morris. It carries the actin gene from \textit{A. nidulans} cloned as a HindIII fragment into pUC18. Plasmid PILJ16 was a gift from I. Johnstone. It contains the \textit{argB} gene from \textit{A. nidulans} cloned as a SalI fragment into pUC8 (Johnstone, Hughes and Clutterbuck 1985). The \textit{A. nidulans} \textit{EMBL4} gene library was a gift from C. M. Lazarus.

**RESULTS**

**Isolation of transformants resistant to 2-thioxanthine:** Reconstruction experiments with regenerating protoplasts made from a strain which is 2-thioxanthine-resistant (\textit{uapA24 biA1}) showed that it was easy to detect green colonies on the background of wild-type (\textit{biA1}) yellow conidiating colonies. A strain carrying a complete deletion of the \textit{amdS} gene \textit{(amdS368 amd118 amdA7 niaA4 biA1)} (Hynes 1979) was transformed with plasmid pAMD21 carrying an intact \textit{amdS} gene and bearing no sequences homologous to the genomic DNA of the recipient strain. Transformants were selected on acetamide as nitrogen source in the presence of 2-thioxanthine (0.1 mg/ml final concentration). Among approximately 12,000 transformants, 34 were 2-thioxanthine-resistant, \textit{i.e.}, showed green conidia. These results represent the aggregate from three different experiments. In experiment 1, one 2-thioxanthine-resistant colony was isolated among 3400 \textit{amdS}° colonies; in experiment 2, the yield was one 2-thioxanthine-resistant colony among 2000 \textit{amdS}° colonies; and in experiment 3, we obtained 32 2-thioxanthine-resistant colonies among 6500 \textit{amdS}° colonies. In this last experiment, 30 different transformation mixtures were plated onto separate Petri dishes. In a similar experiment, a strain carrying a point mutation in the gene coding for ornithine carbamyl-transferase (\textit{argB2 biA1}) was transformed with 200 μg of plasmid PILJ16 carrying an intact \textit{argB} gene (Johnstone, Hughes and Clutterbuck 1985). Transformants were selected on ammonium d-tartrate as nitrogen source in the absence of arginine and in the presence of 2-thioxanthine. Among the 10,000 transformants recovered, no 2-thioxanthine resistant colonies were found.

The 34 2-thioxanthine-resistant transformants were purified and tested on the media described in Table 1 in parallel with a number of 2-thioxanthine sensitive transformants as controls. All 34 were shown to grow on acetamide and acrylamide as nitrogen sources and to maintain their 2-thioxanthine resistance. It was striking, however, that all belonged phenotypically to the \textit{uapA} class. No \textit{hxA}, \textit{hxB}, \textit{cnx} or \textit{uay} mutants were found.

**Analysis of the integration events in transformants showing the \textit{uapA} phenotype:** Six transformants showing the \textit{uapA} phenotype were analyzed by Southern blot. The Southern blot includes the two transformants obtained in experiments 1 and 2 and four transformants obtained in experiment 3. Each of these four transformants was isolated from a different Petri dish and thus originated in a different transformation mixture. Figure 1 shows an EcoRI digest of DNA from these transformants. All transformants showed a complex but nearly identical pattern when probed with pAMD21. This plasmid does not hybridize with the residual acetamidase sequences. These can be detected only when probing with plasmid pBSR2 (Tilburn et al. 1983); all transformants retain, as expected, the residual flanking sequences of the \textit{amdS}.
formants T1 and T19 were selfed as described in Materials and Methods and progeny were analyzed on different nitrogen sources (acetamide, acrylamide, hypoxanthine and hypoxanthine/allopurinol). Three distinct classes of progeny were found: progeny with reduced amdS activity (T1.5, T19.3, T19.4), progeny which have lost amdS activity (T1.1, T1.2, T1.3, T19.1, T19.2, T19.5). No uapA+ progeny, i.e., those sensitive to allopurinol were recorded. T33 is another original 2-thioxanthine-resistant transformant and ts is a 2-thioxanthine-sensitive transformant. wt indicates wild type (biA1) and r is the recipient strain (amds368amd118amd7niiA4biA1).

locus (data not shown). pAMD21 detects five main bands. Integration should have given a 6.3-kbp band corresponding to the size of the plasmid, plus two bands of the flanking sequences. In each case, two additional bands could be seen, implying a second integration event. This interpretation was supported by Southern blot analysis of DNA digested with XhoI, a restriction enzyme which does not cut pAMD21. Two high molecular weight bands were visible (data not shown). It is difficult, however, to understand why a second integration event should always be associated with the integration event which led to uapA inactivation.

**Meiotic analysis of selected transformants:** A. nidulans is a homothallic organism and we have shown that selfing leads to rearrangement of integrated sequences (Tilburn et al. 1983; Durrens et al. 1986). Analysis of selfed cleistothecia of transformants T1 and T19, both being 2-thioxanthine-resistant, showed three classes of progeny. Class 1 is indistinguishable from the parental transformants. Class 2 (including T1.5 and T19.3) shows reduced growth on acetamide and loss of growth on acrylamide, implying a reduction in the number of copies of the amdS gene. Finally, class 3 (including T1.1 and T19.2), like the recipient strain, does not grow on acetamide or acrylamide, showing a complete loss of amdS function. These results, shown in Figure 2, corroborate those described earlier (Tilburn et al. 1983; Durrens et al. 1986). All progeny maintain the uapA+ phenotype. Figure 2 shows the distribution of progeny for two selfed cleistothecia isolated from transformants T1 and T19.

Southern blot analysis of selected selfed progeny is shown in Figure 3A. EcoRI digests, probed with pAMD21, showed a substantial rearrangement and simplification of integrated copies. It was obvious that among the progeny analysed, all strains showing the amdS+uapA+ phenotype retained the 7- and 9-kbp bands, and that progeny showing the amdS+uapA+ phenotype have a single band, of 12-13 kbp, which is not present in digests from the original transformants. XhoI digests, probed with pAMD21, were in agreement with these results. A single high molecular weight band is maintained in the amdS+uapA+ progeny but is replaced by a new, higher mobility, band in the amdS+uapA+ phenotype (results not shown). Progeny from both T1 and T19 showed identical band patterns in agreement with their phenotypes. The significance of this result will be apparent later.

**The amdS gene maps at the uapA locus:** Transformants T1 and T19 and their meiotic progeny T1.5 and T19.3 (amdS+uapA+) were crossed with strain ya2 amdS320 amd118 amd7 niiA4 pantob100 biA1 (amdS+uapA+). The results are shown in Table 2A. While the presence of amdS+uapA+ progeny was ex-
TABLE 2
Mapping the integration of amds sequences in 2-thioxanthine-resistant transformants

<table>
<thead>
<tr>
<th>Phenotype of progeny</th>
<th>T1</th>
<th>T1.5</th>
<th>T19</th>
<th>T19.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>amds+uapA*</td>
<td>78</td>
<td>120</td>
<td>101</td>
<td>96</td>
</tr>
<tr>
<td>amds+uapA</td>
<td>45</td>
<td>108</td>
<td>95</td>
<td>54</td>
</tr>
<tr>
<td>amds+uapA-</td>
<td>21</td>
<td>0</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>amds-uapA*</td>
<td>144</td>
<td>228</td>
<td>213</td>
<td>133</td>
</tr>
</tbody>
</table>

A. Cross

<table>
<thead>
<tr>
<th>Phenotype of progeny</th>
<th>T15</th>
<th>T19.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>uapA*</td>
<td>138</td>
<td>140</td>
</tr>
<tr>
<td>uapA+</td>
<td>138</td>
<td>140</td>
</tr>
</tbody>
</table>

B. Cross

<table>
<thead>
<tr>
<th>Phenotype of progeny</th>
<th>T15</th>
<th>T19.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>uapA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uapA-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

T1 and T19 are original amds"uapA" transformants obtained with an amds368 amdl8 amdA7 niiA4 bial strain. T1.5 and T19.3 are selected selfed progeny from the above transformants sharing an amds"uapA" phenotype.

pected, given the results of the selfing experiment, in no case were amds"uapA" recombinants found. This is strong evidence that functional amds sequences have integrated into, and inactivated, the uapA locus. The number of amds"uapA" progeny seems lower in the crosses involving the T1.5 and T19.3 progeny than in those involving the original transformants. This might not be significant, as excision events can well be premeiotic and vary from one cleistothecium to another (Skelker et al. 1988; Tilburn 1988). Alternatively, this may be related to the reduced number of copies in the progeny, which will result in an increase in the meiotic (or premeiotic) stability of the inserted sequences.

T1.5 and T19.3 were also crossed with strain ya2 uapA24 pantb100. No uapA* recombinants were recovered among the progeny analyzed (Table 2B), giving additional evidence for amds integration at the uapA locus.

Cloning of uapA: The evidence indicates that at least one copy of the amds gene has integrated into the uapA gene, resulting in its inactivation. The EcoRI restriction pattern of progeny T19.3, probed with pAMD21 (Figure 3A), with the 3.6-kbp EcoRI-SmaI fragment comprising the amds sequences of pAMD21, and with pUC9 (results not shown), demonstrates that one copy of the amds gene from pAMD21 has recombined heterologously, presumably in the uapA locus, and that this event has taken place via sequences of the amds gene or flanking. A. nidulans sequences and not via plasmid sequences.

Genomic DNA from T19.3 and T1.5 was used to transform competent E. coli (DH1) cells as described by Johnstone, Hughes and Glatterück (1985). Ten ampicillin-resistant clones were obtained from T19.3 DNA and three from T1.5 DNA. All 13 rescued plasmids were larger than pAMD21 and, when cut with restriction enzymes EcoRI, SalI or PstI, gave identical band patterns, different from those of pAMD21 cut with the same enzymes (results not shown). One of the plasmids, named pAN501, was selected for further study. Restriction analysis (not shown) of pAN501 showed that most Aspergillus sequences downstream from the amds gene, which were present in the original transforming plasmid pAMD21, had been lost. The ClaI site, which is 189 nt before the end of the amds open reading frame (Corrick, Twomey and Hynes 1987), is conserved. Plasmid pAN501 contains a functional amds gene, because it transforms a strain carrying the amds368 deletion. The same plasmid does not transform a strain carrying a uapA24 mutation to prototrophy.

pAN501 was used to probe a nitrocellulose membrane identical to the one shown in Figure 3A (Figure 3B). The results strongly suggest that the 10-kbp band which is detected by pAN501 in uapA" strains represents intact uapA sequences. This band is also present in 2-thioxanthine-sensitive transformants but is replaced by two bands of 7 and 9 kbp in 2-thioxanthine-resistant transformants and in progeny of class 2. The integration of pAMD21 (6.3 kbp) into sequences represented by a 10-kbp EcoRI restriction fragment introduces a plasmid EcoRI site which splits this fragment into two new fragments of total size 16.3 kbp. This is clearly visible in the simplified progeny T19.3 and T1.5. Progeny T19.2 and T1.1 (class 3) seem to have lost plasmid sequences, including the EcoRI site, and thus a single band (12-13 kbp) could be seen that was different from those in original transformants, progeny of class 2 and wild-type strains. However, this excision event, which took place in T1.1 and T19.2 and led to an amds"uapA" phenotype, is different from the mitotic excision event which yielded plasmid pAN501. From the Southern blot analysis presented in Figures 1 and 3 (band pattern, size and intensity) and the structure of the rescued plasmid pAN501, we propose that the integration-excision events which led to the isolation of plasmid pAN501 are those shown in Figure 4.

An A. nidulans λ EMBL4 gene library was probed with pAMD21 and pAN501 and clones which hybridised with only pAN501 were selected. Two such clones gave phages LAN501 and LAN502; the first completely overlapping the second. LAN501 carries a 15.5-kbp insert, including a 10-kbp EcoRI fragment (which hybridizes with pAN501) equivalent to the one detected earlier in Southern blot analysis and which
Figure 4.—A possible integration-excision pathway for the inactivation of uapA by the transforming plasmid pAMD21 and the subsequent generation of rescued plasmid pAN501. The white bar indicates the uapA region, the black bar indicates amdS sequences, and lines indicate pUC9 sequences. Excision points are shown with arrows (Bg = BglII, Xb = XbaI, S = SalI, E = EcoRI, C = ClaI, Sm = SmaI).

should correspond to intact uapA sequences (results not shown). Figure 5 shows the relationship of the pAN501 and LAN501 physical maps.

LAN501 was used in co-transformation experiments with pAMD21 to transform an amdS⁻ universe-¹ double mutant (amdS368 amdJ18 amdA7 niA4 uapA24 biAl pantoB100). Among 40 amdS⁺ transformants selected, 10 were also uapA⁺, suggesting that LAN501 carries a functional uapA gene.

The 4.2- and 3.5-kbp BglII-SalI restriction fragments, contained in the 10-kbp EcoRI fragment of LAN501, were subcloned into the bluescript (KS⁺) M13 vector, and the new plasmids were named pAN502 and pAN503 respectively. In analogous cointegration experiments, pAMD21 and an amdS⁻ universe-¹ double mutant, only pAN503 gave transformants with a uapA⁺ phenotype, while no such transformants were found with pAN502. The high frequency of cointegration with pAN503 (30 uapA⁺ out of 40 amdS⁺ transformants) suggested that a complete functional uapA gene is included in the 3.6-kbp BglII-SalI fragment of this plasmid (Figure 5). This was subsequently confirmed by Northern blot analysis (see below).

Characterization of the uap-100 mutation: We have available four cis-acting mutations affecting the expression of uapA. The best studied, uap-100, was discussed in the Introduction. Three others, uap-302, uap-310 and uap-349 (D. GORTON and C. SCAZZOCCHIO, unpublished results), have no phenotype except that they result in a bypass of the areA102 mutation. Southern blot analysis of genomic DNA from all four mutants, probed with pAN503, revealed that only uap-100 results in a detectable change in the restriction pattern (Figure 6). This change corresponded to an approximately 200-bp insertion or duplication internal to the 1.8-kbp BglII-PstI fragment (Figure 5).

Genomic DNA from uap-100 was digested with EcoRI, ligated to a λ EMBL4 vector and packaged in vitro. A collection of 4000 clones from the uap-100 library was screened with pAN503 and hybridizing clones were isolated. One of them, LAN510, was used to further characterise the uap-100 mutation. Restriction analysis of a uap-100 subclone including a 3.6-kbp BglII-SalI fragment indicated that this mutation is an approximately 150-nt direct duplication including an NheI site (Figure 5).

The BglII-PstI region, including the uap-100 mutation, was subcloned into M13 vectors and sequenced in parallel with the same region from a wild-type strain. The results showed that uap-100 is a 139-bp direct duplication in the putative cis-acting region of
The \textit{uapA} Permease of \textit{A. nidulans} \textbf{347}

\textbf{FIGURE 5.}—Physical map of \textit{A. nidulans} sequences in vectors pAN501, LAN501 and pAN503. pAN501 includes a complete functional \textit{amdS} gene but does not have \textit{uapA} transforming activity. The black bar indicates the \textit{amdS} coding region while the white bar shows rescued sequences from the \textit{uapA} locus. LAN501, selected from an \textit{A. nidulans} library using pAN501 as a probe, contains a functional \textit{uapA} gene. The white bar indicates sequences of the \textit{uapA} region. pAN503, constructed by cloning the \textit{BglII-SalI} fragment from LAN501 into bluescript (ks') M13 vector (striped bar), includes a complete \textit{uapA} gene and its flanking sequences (white bar). The proposed borders of the \textit{uapA} transcript are shown. The region in the cis-acting area of \textit{uapA} duplicated in the \textit{uap-100} mutation (black bar) and the proposed sequence (CTTTTTTGG) involved in the \textit{uapA/amdS} integration-excision event are also shown (Sm = SmaI, Bg = BglII, Xb = XbaI, S = SalI, E = EcoRI, C = Clal, B = BamHI, N = NheI, H = HincII).

\textit{uapA}, 115 bp upstream from the start of translation (Figure 7). No other change was found in the DNA of this region. The complete \textit{uapA} sequence will be published separately.

To confirm that this duplication of 139 bp causes the \textit{uap-100} phenotype, we transformed an \textit{areA102} strain \textit{fusA1 areA102 pyroA4}, which cannot grow on uric acid as sole nitrogen source (\textit{ARST} and \textit{SCAZZOCCHIO} 1975) with 5 \(\mu\)g of M13 single-stranded subclones including the \textit{BglIII-PstI} region, using both wild-type and \textit{uap-100} DNA, and selected for growth on uric acid as sole nitrogen source. Only the \textit{uap-100} subclone gave transformants able to grow on uric acid. Four such transformants were further tested on different nitrogen sources and shown to have an \textit{areA102 uap-100} phenotype (\textit{ARST} and \textit{SCAZZOCCHIO} 1975). Southern blot analysis of DNA from these transformants, probed with pAN503, showed that all are the result of sequence replacement events, \textit{i.e.}, all showed the \textit{uap-100} duplication when digested with \textit{BglII} and \textit{PstI} (results not shown). This result is in agreement with results obtained from single-stranded transformations and subsequent gene replacement events using other \textit{cis}-acting regulatory mutations (V. SOPHIANOPOULOU, G. DIALLINAS and C. SCAZZOCCHIO, unpublished data). The technique of sequence replacement by single-stranded transformation follows

the methodology of GYOYON and FAUGERON (1988) for the fungus \textit{Ascobulus immersus}.

\textbf{The \textit{uapA} transcript in wild-type and \textit{uap-100} strains.} Northern and dot blot analyses of poly(A)+ RNA from wild-type and \textit{uap-100} strains are shown in Figure 8. A \textit{uapA}-specific transcript of approximately 2.1 kbp is transcribed from \textit{BglII} to \textit{PstI} (see Figure 5). This direction of transcription is consistent with the \textit{uap-100} mutation having occurred 5' to the gene. The mRNAs present in the wild-type and \textit{uap-100} strains have identical sizes, within the limits of the Northern blot analyses. The 5' ends lie for both mRNA species within the 1.8-kbp \textit{BglII-PstI} region. Both transcripts end before the \textit{XbaI-SalI} 0.8-kbp region (data not shown).

The relative amounts of the \textit{uapA} transcript in wild-type and \textit{uap-100} strains grown under inducing and non-inducing conditions were estimated by dot-blot densitometry and normalized after hybridization of the same membranes with plasmid pSF5 containing the actin gene (\textit{ARST} 1978; \textit{SCAZZOCCHIO and ARST} 1979). The \textit{uap-100} mutation results in a 5-fold increase of the \textit{uapA} transcript in mycelia grown under noninducing conditions and in an 8-fold increase in mycelia grown under inducing conditions. Thus, the \textit{uap-100} mutation increases both the basal level of \textit{uapA} expression, resulting in an apparent constitutivity, and the net inducibility of the gene. These results are also in agreement with those found earlier in uptake studies (\textit{ARST} and \textit{SCAZZOCCHIO} 1975; \textit{SCAZZOCCHIO} and \textit{ARST} 1978).

\textbf{DISCUSSION}

The results presented here describe the inactivation cloning and initial characterization of the uric acid-xanthine permease gene (\textit{uapA}) in \textit{A. nidulans}.
These results show that heterologous integration is not random in *A. nidulans*. The 2-thioxanthine technique could have detected integration events at nine well characterized genes. Not only does the *amdS* gene integrate into just one of these loci (*uapA*), but it also seems to integrate in a very specific manner. All 2-thioxanthine-resistant transformants were nearly identical and all rescued plasmids and meiotic excision events (T1.1 and T19.2) were, within the limits of restriction and Southern blot analyses, identical. The integration-excision events between the *uapA* and *amdS* sequences seem to result from the possibility of a specific recombination pathway. In fact, we know that the sequence CTTTTTTTGG is present 3′ in the *amdS* stop codon (CORRICK, TWOMEY and HYNES 1987) and within the *uapA* open reading frame (position +260 from the ATG) (Figures 5 and 7). This sequence is between the *ColI* site within the *amdS* open reading frame and the *XbaI* site distal to the termination codon (Figures 4 and 5). The *ColI* site is conserved and the *XbaI* site is lost in the plasmid pAN501. Thus, this sequence might be involved in the integration and excision events. Sequencing through the borders of the *amdS/uapA* integration (T19.3) and excision (pAN501) products would allow the detailed characterization of this event.

The above observation agrees with previous results (TILBURN 1988) which suggested that transformants comprised distinct classes with respect to the locations of heterologous integration events. Thus, it is not always possible to use selective techniques to isolate specific inactivation events. However, we were particularly fortunate in obtaining integration events in the *uapA* gene. Because *uapA* mutants are leaky, due to the presence of at least one other permease able to incorporate uric acid into the cell (ARST and SCAZZOCCHIO 1975), *uapA* cloning by direct complementation would have been extremely laborious. We have observed only one other case of heterologous integration into a known gene: the inactivation of the *wa* gene at very low frequency on several independent occasions using different transforming plasmids. However, integration events at *wa* do not show the uniformity found in *amdS/uapA* heterologous recombination. These results will be reported elsewhere (J. TILBURN and C. SCAZZOCCHIO, unpublished results). It is possible that integration into *wa* depends on special features of the target sequence, at the level of primary or secondary structure, rather than on limited sequence identity.

The excised sequences from appropriate transformants allowed us to obtain a genomic *uapA* clone. This in turn was used to characterize a cis-acting mutation with an interesting phenotype (*uap-100*). The most striking characteristic of this mutation is that, while maintaining a strict dependence on the *waY* and *areA* gene products, it increases the level of the *uapA* transcript 8-fold. This increase is greater than that found with uptake studies (ARST and SCAZZOCCHIO 1975; SCAZZOCCHIO and ARST 1978). This can most easily be explained by assuming a limited capacity to translate *uapA* mRNA, which may or may not be linked to a limited capacity to incorporate *uapA* permease molecules in the membrane. The other interesting features of *uap-100* are that it bypasses the need for induction and leads to *uapA* expression in an *areA102* background. How does a 139-bp duplication result in this phenotype? It is possible that the *uap-100* duplication includes the *areA* or the *waY* binding sites or both. A duplication of the *areA* binding site could account for the suppression of the *areA102* phenotype. The phenotype of this and other *areA* mutations implies that the binding sites for *areA* are not unique, but that different sites respond differentially to modified *areA* gene products (ARST and COVE 1973; ARST and SCAZZOCCHIO 1975; AL-TAHIO, SEALY-LEWIS and
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Scanzocchio 1984). If the areA102 mutation results in a drastic loss of binding specific for the uapA-upstream areA binding site, a duplication of this site might restore efficient binding through by establishing a co-operative effect. A similar cooperative effect might explain why strains carrying the uap-100 mutation strongly express the uapA gene in the absence of induction while still requiring the presence of the uay gene product. In other words, the uap-100 duplication might increase the affinity of the uay binding site for the unliganded form of the uay gene product, as already proposed by Scanzocchio and Arst (1978).

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LITERATURE CITED


FIGURE 8.—Northern and dot blot analyses of the uapA transcript in a wild-type and a uap-100 background. Poly(A)* RNA was extracted in every case from 1 mg of total RNA as described in MATERIALS AND METHODS. The whole preparation of poly(A)* RNA was loaded onto a 1% agarose gel. The yield from different extractions was not uniform and varied between 2 (for WT, NI and I) and 10 μg (for uap-100, NI and I). After fractionation, the mRNAs were transferred onto nitrocellulose which was hybridized with a uapA-specific M13 single-stranded probe (containing the Bglll-PstI 1.8-kbp fragment shown in Figure 5) as described in MATERIALS AND METHODS. (A) shows a 6-hr exposure and (B) a 60-hr exposure of the autoradiograms at −80°. The size of the uapA-specific mRNA is indicated. The membrane was then dehybridized and probed with plasmid pSF5 which detects the actin mRNA. The actin controls reflected the differences in loading (results not shown). Taking into account these differences, the uap-100 mutation results in a ~5-fold increase of noninduced uapA mRNA and an ~8-fold increase of the induced uapA mRNA. A more accurate estimate was obtained by dot blots shown in (C). The mRNAs were loaded in the same order as in Figure 8B. The densitometry readings, normalized against the actin concentrations and giving an arbitrary value of 1 to the WT NI, were 2.5, 5 and 20 for WT 1, uap-100 NI and uap-100 I, respectively. WT indicates the wild-type strain (biA1); uap-100 indicates an isogenic strain carrying this mutation (biA1 uap-100). NI indicates mycelium grown under non-inducing conditions and I indicates mycelium grown under inducing conditions (see MATERIALS AND METHODS).


