Molecular Cloning and Characterization of a Transcriptional Activator Gene, *amyR*, Involved in the Amylolytic Gene Expression in *Aspergillus oryzae*

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A gene, designated *amyR*, coding for a transcriptional activator involved in amylolytic gene expression has been cloned from *Aspergillus oryzae* by screening for a clone that enabled to reverse the reduced expression of the α-amylase gene (*amyB*) promoter. *amyR* encodes 604 amino acid residues of a putative DNA-binding protein carrying a zinc binuclear cluster motif (Zn(II)2Cys8) belonging to the GAL4 family of transcription factors. The *amyR* gene disruptants showed a significant restricted growth on starch medium and produced little of the amylolytic enzymes including α-amylase and glucoamylase compared with a non-disruptant, indicating that *amyR* is a transcriptional activator gene involved in starch/maltose-induced efficient expression of the amylolytic genes in *A. oryzae*. In addition, sequencing analysis found that *amyR*, *agdA* (encoding α-glucosidase), and *amyA* (encoding α-amylase), are clustered on a 12-kb DNA fragment of the largest chromosome in *A. oryzae*, and that *amyR* is about 1.5 kb upstream of *agdA* and transcribed in the opposite direction. Furthermore, transcriptional analysis revealed that the *amyR* gene was expressed in the presence of glucose comparable to the level in the presence of maltose, while the amylolytic enzymes were transcribed at high levels only in the presence of maltose.

**Key words:** *Aspergillus oryzae*; transcriptional activator; amylolytic enzyme; zinc finger motif; gene cluster

*Aspergillus oryzae*, which plays a pivotal role in traditional Japanese fermentation industries, produces copious amounts of glucan hydrolases such as α-amylase (Taka-amylase A), glucoamylase, and α-glucosidase. Production of these enzymes is induced in the presence of starch or maltose oligosaccharides such as maltose, maltotriose, and isomaltose, but not of glucose. It was shown by Northern analyses that the expression of these genes was regulated at the transcriptional level. We have already cloned and sequenced the genes encoding Taka-amylase A (*amyB*),1 glucoamylase (*glaA*),2,3 and α-glucosidase (*agdA*)4 from *A. oryzae*. By comparing these with the promoter sequences of the *A. oryzae* amylolytic genes, three regions designated region I, II, and III were found to be highly conserved. To discover the function of the conserved sequences in starch/maltose induction, fusion genes of amylolytic gene promoters and the *Escherichia coli* uidA, encoding β-glucuronidase (GUS) were constructed and deletion analyses were done in *A. oryzae*.5,6 Consequently, region III is most likely involved in maltose induction as well as high-level expression and region I has a cooperative effect on the efficient expression of the genes with region III. These results indicated that there might exist certain transcriptional activator protein(s) that could interact with the conserved sequence, region III. Independently, Nagata et al.9 found a nuclear protein, AnCP, which was able to bind in vitro a CCAAT sequence in the *amyB* (*iaaG2*) promoter, from *Aspergillus nidulans* and suggested that a CCAAT sequence was required for maltose induction and high-level expression for the *amyB* gene. Recently, however, Kato et al.10 has shown that by mutantional experiment the CCAAT sequence is involved in high-level expression for the *amyB* gene but not in maltose induction. We also obtained the same result by site specific deletion of the CCAAT sequence, which is referred region IIIb because it was located adjacent to region III, in the *agdA* promoter.6 Therefore, the CCAAT sequence in the amylolytic gene promoters and a CCAAT binding protein such as AnCP could be responsible for high-level expression of the amylolytic genes, but other unknown protein factors required for maltose induction, possibly by interacting with region III-binding protein, would exist in *Aspergillus*

On the other hand, the observation that region III may be a functional *cis*-element essential for maltose induction and high-level expression led us to apply

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the sequence for construction of a further high expression promoter. Indeed, introduction of the multiple copies of the fragment comprising region III into the agdA promoter resulted in a remarkable increase in promoter activity.\textsuperscript{11} In addition, using the improved promoter, a high yield of $\alpha$-glucosidase and nucleotide S1, the production levels of which are very low by nature because of their own gene expression level in wild-type A. oryzae, could be gained.\textsuperscript{11} Interestingly, in such transformants to which the vector containing the improved promoter was introduced, the expression of the amyB and glaA genes was significantly reduced, simultaneously. Similar observations were previously obtained in the glaA or agdA multiple-copy transformants.\textsuperscript{3,12} Since region III is conserved in the promoter in the amyB, glaA, and agdA genes, provided that trans-acting regulatory protein(s) would interact with the sequence, those phenomena could be caused by titration of the common activator protein(s). If this is the case, we supposed that introduction of multiple copies of the putative gene encoding a transcriptional activator would suppress the titration. Hynes \textit{et al.} previously observed that multiple copies of the promoter region of \textit{A. nidulans amdS} directed the reduction of several genes the expression of which is controlled by a common activator gene, amdR,\textsuperscript{13} and that multiple copies of the amdR could actually reverse the titration phenomenon (anti-titration).\textsuperscript{14} In this context, it would be possible to isolate a transcriptional activator gene involved in the regulation of the amylolytic gene expression from an \textit{A. oryzae} gene library by screening a clone that enables to reverse the titration event. Here we report the successful cloning, nucleotide sequencing, and characterization of the transcriptional activator gene, designated \textit{amyR}.

\section*{Materials and Methods}

\textit{Media and growth conditions.} Minimal medium (MM) used in this study was essentially described by Cove\textsuperscript{15} supplemented with appropriate nutrient requirements. The carbon source was 1\% maltose, starch, or glucose. Modified YPD medium, consisting of 1\% glucose, 1\% polypeptide, and 0.5\% yeast extract, was used as the complete medium (CM). For plate assay for $\beta$-galactosidase, 20 or 40 ppm of 5-bromo-4-iodo-3-indolyl-$\beta$-galactopyranoside (X-Gal, Wako Pure Chemicals) was added to the MM.

Basically, \textit{A. oryzae} and \textit{A. nidulans} were grown at 30\(^\circ\)C and 37\(^\circ\)C, respectively.

\textit{Strains and plasmids.} \textit{A. oryzae} RIB40 was used as a wild-type genomic DNA donor for construction of an \textit{A. oryzae} genomic library. As hosts for transformation experiments were used \textit{A. oryzae} M-2-3 (w, argB)\textsuperscript{16} and \textit{A. nidulans} WG355 (biA1, argB2, bga0),\textsuperscript{17} which was kindly provided by G. Turner (University of Sheffield).

\textit{A. nidulans} KG315 expressing \textit{Escherichia coli} $\beta$-galactosidase under the control of the amyB promoter (d315) was obtained as follows. First, 5'-untranslational region (315 nucleotides) of the amyB containing regions I, II, and III but not a typical CCAAT sequence and the nucleotides corresponding to the first 3 amino acids of the amyB were inserted into a BamHI site of the plasmid pAN923-41B\textsuperscript{18} to give pAN923GKd315 (Gomi, K., and Turner, G., unpublished data), which directs lacZ expression under the control of the amyB promoter. Then pAN923GKd315 was digested with XbaI and SpI and removed the \textit{A. nidulans} argB followed by insertion of XbaI-Sphi fragment containing the \textit{A. nidulans} scC from pUSC.\textsuperscript{19} The resultant plasmid was digested at a unique HindIII site located within the scC fragment and was then used for transformation of \textit{A. nidulans} WG355. Two transformants were obtained as colonies developing a blue color on the agar-solidified MM containing X-Gal and maltose. Finally, a transformant, KG315, was selected by Southern hybridization analysis that showed a single copy of the introduced DNA integrated at the resident scC locus in \textit{A. nidulans}. \textit{A. nidulans} FGSC773 (wA3, pyrG89, pyroA4) was obtained from the Fungal Genetics Stock Center (University of Georgia) and was used for a sexual cross with KG315 to construct a recipient strain, KG40 carrying argB+pyrG double auxotrophic markers. The sexual cross of \textit{A. nidulans} was done according to Pontecorvo \textit{et al.}\textsuperscript{20}

\textit{Escherichia coli} DH5a (supF44, hisdR17, recA1, endA1, gyrA96, thi-1, relA1, lacU169/Phi80lacZ-M15) was used for propagation of plasmid DNAs.

Plasmid pDHG25, kindly provided by A. J. Clutterbuck (University of Glasgow), was used for construction of an \textit{A. oryzae} genomic library. pDHG25 carries the \textit{AMAI} fragment that confers autonomously replicating ability on the vector DNA and increases the transformation efficiency.\textsuperscript{21} In addition, \textit{AMAI}-based pDHG25 was used for co-transformation experiments where subcloning of the isolated DNA fragment was done.

pGFP-N1 (Clontech) was used to construct multiple tandem copies of the fragment containing region III. Briefly, a 0.6-kb fragment comprising 12 tandem copies of region III and CCAAT sequence (IIIb) was isolated from PagdA142\textsuperscript{22} by digesting with \textit{XhoI} and \textit{EcoRV} and then inserted between the \textit{XhoI}-SmaI site of pGFP-N1. Then the resulting plasmid was digested with \textit{BglII} and \textit{BamHI} to recover the 0.6-kb fragment, which was cloned again into the \textit{BamHI} site of the resulting plasmid. When the head-to-tailed ligation would occur, a DNA fragment ranging from 1.2-2.4-kb in length could be isolated by digestion of the constructed plasmid with \textit{BglII} and \textit{BamHI}. Following reiteration of such manipulation, an approximately 9-kb fragment comprising
144 copies of tandem repeated region III was recovered and used for transformation of *Aspergillus* to examine whether or not the titration phenomenon occurs.
pSal23 and pDBJ1 were used for transformation of *Aspergillus* based on arginine and uracil prototrophy, respectively.
For gene disruption, a plasmid, pΔamyR2, was constructed by ligating an *XbaI* fragment of the argB gene isolated from pAN923-41B to a pUC19-based plasmid containing a truncated *amyR* fragment that lacks 0.15-kb and 0.7-kb of the coding region from the 5'- and 3'-termini, respectively.

*Construction of the* A. oryzae *genomic DNA library*. Genomic DNA derived from *A. oryzae* RIB40 was partially digested with Sau3A1 and was fractionated through a sucrose density centrifugation. Approximately 4 to 10-kb digested DNA fragments were fractionated and ligated to the BamHI site of pDHG25. Total independent clones based on pDHG25 in the genomic DNA library were approximately 30,000, which could cover 99.5% of the genome of *A. oryzae*.

*Transformation experiments*. Transformation of *E. coli* and *Aspergillus* was done by the method of Hanahan and Gomi et al., respectively.

*Southern blot analysis*. An *A. oryzae* wild-type strain, RIB40 and the *Aspergillus* transformants were grown in 10 ml of CM with shaking. Genomic DNA was prepared by pulverization of the mycelium in liquid nitrogen with a pestle and mortar and purified as described previously. Genomic DNA was digested with restriction enzymes followed by separation on 0.8% agarose gel electrophoresis and then transferred onto a positively charged nylon membrane (Hybond-N+, Amersham). Probe labeling and detection were done by use of ECL labeling and detection system (Amersham). Hybridization was done according to the manufacturer's instruction.

*Northern blot analysis*. *A. oryzae* RIB40 was grown in 50 ml of MM containing 1% maltose or glucose at 30°C for 20 hr. Then total RNA was isolated from pulverized mycelium by the acid guanidinium-phenol-chloroform (AGPC) method of Chomczynski and Sacchi using ISogen (Nippon Gene). Fifteen μg of total RNA was separated on a formaldehyde-denatured agarose gel and transferred onto a Hybond-N nylon membrane (Amersham) followed by cross-linking by UV irradiation. DNA probes were 32P-labeled with a Ready-to-Go labeling system (Pharmacia Biotech). Hybridization was done at 45°C in 6×SSC, 5×Denhart's solution, 0.5% SDS, 100 μg/ml salmon sperm DNA, and 50% formamide for 16 hr. Washing was done at 45°C in 2×SSC, 0.1% SDS twice for 15 min and then in 1×SSC, 0.1% SDS for 15 min at 45°C.

*Isolation of cDNAs of the* amyR *gene*. Poly(A)+ RNA was purified from total RNA isolated with Oligotex-dT30 (Takara Shuzo). cDNAs of 5' and 3'-terminal ends of the gene were isolated by means of 5'- and 3'-RACE (rapid amplification of cDNA ends) with Marathon cDNA amplification kit (Clontech). PCR amplification in 5'-RACE was done with first denaturation at 94°C for 1 min, and in 5 cycles with 94°C, 30 s; 72°C, 3 min, then in 5 cycles with 94°C, 30 s; 70°C, 3 min, and finally in 25 cycles with 94°C, 20 s; 68°C, 3 min. 3'-RACE was done with first denaturation at 94°C for 1 min, and then in 30 cycles with 94°C, 30 s; 60°C, 30 s; 68°C, 3 min. The amplified DNA fragments were isolated through agarose gel electrophoresis using GeneClean II (Bio101) and then inserted into pT7Blue (Novagen) to be sequenced.

*Nucleotide sequencing*. The nucleotides of the isolated DNA were sequenced by the dideoxy chain termination method using a BigDye terminator cycle sequencing kit (Perkin Elmer) with an automated DNA sequencer (Perkin Elmer ABI PRISM 310A). DNA sequences were analyzed with the DNASIS (Hitachi Software Engineering) and Sequencher (Tejbin) programs. Similarity searches in database were done using the National Center for Biotechnology Information (NCBI) BLAST software.

*Enzyme assays*. *A. oryzae* was grown in MM containing 1% dextrin and 0.5% polypeptide and culture broth was assayed for α-amylase, which was done as described by Adachi. For β-galactosidase assay, approximately 106 conidia of the *A. nidulans* transformants were inoculated into 10 ml of MM+ 1% maltose, grown at 37°C for 28 hr with shaking, and then harvested. Preparation of cell-free extracts of the transformants was done as described by Tada et al. β-Galactosidase activity of the cell-free extract was measured according to Miller. Protein was measured by the method of Lowry et al., using bovine serum albumin as a standard.

*Results*

*Reduced expression of the* amyB::lacZ *fusion gene by the introduction of multiple copies of region III* We previously demonstrated that the transcriptional levels of the *amyB* and *glA* genes were significantly reduced in the transformant, which overproduced α-glucosidase under control of the improved promoter containing multiple copies of region III and CCAAT sequence (region IIIb). This phenomenon might be caused by titration of the common regulatory protein(s) interacting with region
III and/or IIIb. Since we focused on a putative
region III-binding protein in this study, we examined
whether a similar effect could be observed when mul-
tiple copies of region III were introduced into A.
nidulans expressing E. coli lacZ under the control of
the amyB promoter (d315), which contains region III
but lacks a typical CCAAT sequence. The plasmid
harboring 144 copies of region III and IIIb construct-
ed as described in Materials and Methods was in-
troduced into an A. nidulans KG40 by cotransforma-
tion with pDBJ1. Among the uracil prototrophic
transformants, several clones were found to show a
poor growth and display a faint blue coloration on
the agar-solidified MM containing X-Gal and maltose.
Southern blot analysis of the transformants
showed that a larger amount of region III was in-
tegrated in the genome of the strains growing poorly
than in the genome of ones showing normal growth.
In addition, the more copies were integrated, the
less the activity of β-galactosidase was observed.
(Table 1, Fig. 1) These results indicate that introduc-
tion of multiple copies of region III may titrate a
putative regulatory protein which binds to region III
sequence specifically and is involved in maltose-in-
duced transcription of α-amylase gene, although
the possibility for titration of a CCAAT-binding protein
could not be absolutely ruled out. Furthermore, also
in A. nidulans similar regulation circuit involved in
amylolytic gene expression would operate as in A.
oryzae.

**Cloning of the putative transcrip
tional activator gene by screening for clones that suppress
the titration phenomenon**

Assuming that the regulatory protein which binds
to region III may be titrated by the introduction of
multiple copies of region III, we supposed that the
isolation of the putative gene encoding such a trans-
criptional activator could be accomplished by
screening the clones suppressing the reduced expres-
sion of β-galactosidase. An A. nidulans transformant
144-2, in which the titration effect was observed most
seriously, was chosen as a host for shot-gun cloning
of the activator gene. Approximately 30,000 clones of

**Table 1. β-Galactosidase Activity of the Transformants with
Multiple Copies of Region III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity* (nmole/min/mg-protein)</th>
<th>Ratio**</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG40</td>
<td>20058</td>
<td>1.0</td>
</tr>
<tr>
<td>144-1</td>
<td>7054</td>
<td>0.35</td>
</tr>
<tr>
<td>144-2</td>
<td>2096</td>
<td>0.10</td>
</tr>
<tr>
<td>144-6</td>
<td>3963</td>
<td>0.20</td>
</tr>
<tr>
<td>144-10</td>
<td>2607</td>
<td>0.13</td>
</tr>
<tr>
<td>144-12</td>
<td>19816</td>
<td>0.99</td>
</tr>
<tr>
<td>144-15</td>
<td>3384</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* β-Galactosidase activity of each strain was measured as described in
Materials and Methods.

The A. oryzae gene library based on pDHHG25 were
divided into 22 pools, each of which contained
1,500–2,000 independent clones. The host strain,
144-2, was transformed with 20 μg of DNA prepared
from each pool and regenerated to conidiate
sufficiently on the MM medium containing 1% glu-
cose. Then the conidia formed were replicated onto
the selection agar medium containing X-Gal and mal-
tose. Consequently, among approximately 20,000
transformants obtained, two candidate clones, desig-
nated GL7-1 and GL20-1, were isolated. These two
isolates showed a good growth and displayed a in-
tense blue coloration as well as KG315 or KG40 on
the selection medium. To examine whether the
phenotype of the isolates was actually caused by the
introduced DNA fragment, plasmids were recovered
from their total cellular DNA and re-introduced into
144-2. A plasmid rescued from GL20-1, designated
pGL20, could reverse the reduced growth and β-
galactosidase productivity, but that from GL7-1
could not. Southern blot analysis of GL7-1 showed
that the number of region III copies decreased in
comparison with that of 144-2 (data not shown),
probably because of excision of region III during the
transformation process.

The plasmid, pGL20, contained an approximately
7-kb DNA fragment and was digested with various
restriction endonucleases to construct the restriction
map of the cloned DNA. Based on the restriction

**Fig. 1. Southern Blot Analysis of the A. nidulans Transformants
Carrying the Multiple Copies of the Region III and CCAAT Sequence.**

Approximately 3 μg of genomic DNA from each transformant
was digested with BamHI and separated by 0.8% agarose gel
electrophoresis, followed by transfer onto a Hybond-N
nylon membrane. Hybridization was done with a 0.6-kb fragment con-
taining 12 tandem copies of region III and CCAAT in Pag-
d4143 as a probe. The blot was probed with the A. nidulans
argB gene derived from pAN923-41B as the control for a
single-copy gene, and is shown at the bottom. Lanes 1–6 corre-

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NII-Electronic Library Service
map, several subclones were inserted in pUC119 and co-introduced with pDHG25 into 144-2 to identify the region capable of suppressing the titration effect. Consequently, a 5-kb SacI-BamHI fragment was found to contain the putative gene of interest, amyR, and then was sequenced.

Molecular characterization of a putative transcription activator gene, amyR

The nucleotide sequence of the gene and the deduced amino acid sequence of the amyR gene product (AmyRp) are shown in Fig. 2. To identify the exon/intron junction, transcription initiation, and end points of the gene, cDNAs isolated by 5' and 3' RACE were cloned and sequenced. The open reading frame (ORF) consists of 1,962 bp encoding 604 amino acid residues and is interrupted by two introns of 66 and 84 bp. The identified introns had general characteristics of filamentous fungal introns; they contained GT and AG at the exon/intron junctions and an internal consensus sequence, 5'-PuCTPuAc-3', required for lariat formation. In the 5'-untranslated region of the amyR gene, two putative TATA sequences are found at -405 and -420, and a typical CCAAT box exists at -253 from the putative translation start point. In addition, there are two CT-rich stretches, one of 36 bp at -209 and the other of 18 bp at -160, between the CCAAT box and the putative start codon. As previously reported, the CT-stretch may be involved in determining the correct initiation of the transcription, and thus a major transcription start point would exist, or just after, the CT-stretch. The sequencing of 12 clones of the cDNAs derived from 5'-RACE revealed that T at -156 in the second CT-stretch is the farthest 5'-end, suggesting that transcription would start near by the sequence. If this is the case, putative TATA sequences would be located more than 200 bp apart from the transcription initiation site, and hence these sequences might not function apparently as a cis-element for transcription. On the other hand, a typical polyadenylation signal sequence, AATAAA, was not found in the 3'-untranslated region of the gene. However, its related sequence, AATAGAA, is present just upstream of a transcription end point, C at +2282.

The nucleotide sequencing of the 5-kb SacI-BamHI fragment revealed that amyR and agdA we previously isolated are clustered and are divergently transcribed from a 1.5-kb intergenic region. This fact prompted us to speculate on the existence of a cluster of the amylolytic genes in the A. oryzae genome. We have already found that A. oryzae RIB40 has three copies of α-amylase genes, designated amyA, amyB, and amyC, all have nearly identical nucleotide sequences in the coding and 5'-upstream regions. Southern blot analysis of the separated chromosomes by pulsed-field gel electrophoresis showed that the agdA gene and one of the α-amylase genes, amyA, were located on the largest chromosome in A. oryzae RIB40. We, therefore, supposed that the amyA gene might exist adjacent to the amyR-agdA gene cluster and then sequenced a 35-kb DNA fragment containing the amyA of a cosmid (F1-F5) isolated previously. As a result, the amyA gene was found to be at 2.5 kb downstream of the agdA coding region, but other amylolytic related genes were not found on the fragment. (Fig. 3)

Deduced amino acid sequence of the transcriptional activator, AmyRp

The ORF of the amyR gene encodes a protein of 604 amino acid residues with a predicted molecular mass of 66.6 KDa. The deduced amino acid sequence of AmyRp revealed the presence of a zinc binuclear DNA-binding motif located near the N-terminus between amino acid positions 28 and 54. This motif has similarity to the zinc finger of the fungal transcriptional activator proteins containing the consensus sequence, Cys-X2-Cys-X6-Cys-X5-a-Cys-X2-Cys-Xp-Cys. Six cysteine residues in the motif ligate two zinc ions to form a pair of fingers. The amino acid alignment of the zinc finger region of AmyRp with those found in other fungal transcriptional activators is shown in Fig. 4A. In addition to almost perfect alignment of six cysteines, the proline, which is shown to be important for DNA binding and function in Gal4p, alanine and lysine residues are conserved in AmyRp. Particularly, the highest similarity in the zinc finger region is observed between AmyRp and Mal63p or its homologs from Saccharomyces cerevisiae. (Fig. 4B) Mal63p is a transcriptional activator protein required for efficient expression of the genes encoding maltose permease and maltase (=α-glucosidase). It is, therefore, intriguing on the evolutionary aspects that high similarity was found in the DNA-binding region of regulatory proteins essential for malto-oligosaccharide use in both of A. oryzae and S. cerevisiae.

In addition to the zinc finger domain, leucine zipper-like heptad repeat motif, Leu-X6-Leu-X6-Leu-X6-Leu, was found at amino acid position 351 in AmyRp. This motif was shown to form coiled-coil structures, through which dimerization of the DNA-binding protein would occur. AmyRp may, therefore, bind to DNA as a homodimer. No clear similarity could be found with other regulatory proteins except the two regions described.

Gene disruption of the amyR

Introduction of multicopy of the amyR gene could reverse the titration phenomenon and a predicted protein structure encoded by this gene showed general characteristics of DNA-binding protein, indicating that the amyR gene is most likely responsible for starch/maltose-induced expression of amylolytic
genes in *A. oryzae*. To confirm this, further experiments were done by targeted disruption of the *amyR* gene. For gene disruption, a plasmid, p*amyR*, was constructed as described in Materials and Methods.
Fig. 3. Partial Restriction Map of the Insert in Cosmid F1–F5 Containing the amyR-aggA-amyR Gene Cluster.

The direction of transcription of the genes is shown by an arrow. Restriction sites are abbreviated as E, EcoRl; K, KpnI; P, PstI; Xh, XhoI. The sequence data of 35 kb of the insert have been submitted to DDBJ/EMBL/GenBank databases under accession number AB021876.

Fig. 4. Comparison of the Amino Acid Sequences Around the Zinc Binuclear Motifs Derived from Fungal Regulatory Proteins.

A. Alignment of the amino acid sequences of the zinc fingers. The amino acid position in the protein is indicated at the start of each sequence. Cys (C) in the zinc fingers is shaded. The amino acid sequences of the proteins are derived from the DDBJ/EMBL/GenBank databases, accession numbers in which are indicated.

B. Amino acid sequence homologies among the regions encompassing the zinc fingers of AmyRp and Mal63p homologs.

Integration was directed by homologous recombination between the truncated amyR gene and the resident gene, by digesting pAmyR2 at the unique Apal site in the truncated gene. When the homologous integration takes place, two truncated copies of the gene separated by plasmid sequences should be generated, one lacking a promoter with the 5′-coding region and the other lacking the 3′-coding region. (Fig. 5A) Transformants were purified by transferring successively on the MM, and tested for growth on the starch-containing medium. Five out of 20 transformants examined grew very slowly on the medium as compared with the recipient strain. Southern blot hybridization analysis of genomic DNA of the representative transformants showed that 2 transformants (ΔamyR2 and ΔamyR3) with poor growth, contain the predicted 4.0- and 7.2-kb fragments, while ΔamyR5 had a 3.6-kb fragment, as did a recipient strain. (Fig. 5B) This and PCR analysis (data not shown) indicated that the amyR gene was indeed disrupted in ΔamyR2 and ΔamyR3, but not in ΔamyR5. Figure 6 shows the growth of the transformants on the starch or starch + glucose medium. On the starch medium, three transformants, ΔamyR2, ΔamyR3, and ΔamyR4 (also an amyR disruptant according to Southern blot analysis), showed significantly poor growth, but ΔamyR5 grew vigorously as well as the recipient strain. In contrast, on the starch medium with glucose, these four transformants showed indistinguishable growth. Furthermore, α-amylase activities of the amyR gene disruptants in the submerged culture were significantly lower (approximately 100-fold decrease) than those of the recipient and non-disruptant. (Table 2) Also, glucoamylase and α-glucosidase activities of the disruptant decreased greatly compared with the recipient (data not shown). These results clearly demonstrate that the amyR gene is responsible for starch/maltose-induced efficient expression of amylytic genes.

Transcriptional regulation of the amyR gene

Since the amyR and aggA genes are transcribed divergently from a 1.5-kb intergenic region as shown in Fig. 3, we supposed that the expression of both genes would be regulated in a similar fashion. To verify this, the transcriptional manner of both genes was examined by Northern blot analysis. Contrary to our expectation, the transcript of the amyR gene was detected irrespective of the carbon sources, but that of the aggA gene was only found in RNA from the cells grown in the presence of maltose, as shown in Fig. 7.

Discussion

We have cloned a putative transcriptional activator gene, designated AmyR, involved in the efficient expression and, probably, in maltose/starch induction of the amylytic genes of A. oryzae by screening for a clone that enables to reverse the titration of the predicted regulatory protein. This gene product, AmyR, has been found to contain a zinc binuclear motif similar to a group of typical fungal transcriptional activators. To our knowledge, this is the first
A Transcriptional Activator Gene, *amyR*, of *Aspergillus oryzae*

![Diagram of gene disruption](image)

**Fig. 5.** Disruption of the *amyR* Gene in *A. oryzae*.


B. Southern blot analysis of the *amyR* gene disruptants and the recipient strain, M-2-3. Approximately 3 μg of genomic DNA was digested with *EcoRV*, electrophoresed, and then blotted onto a nylon membrane. The blot was probed with a 0.8-kb *PstI* fragment containing the zinc finger motif.

**Table 2.** α-Amylase Activity of the *amyR* Gene Disruptants

<table>
<thead>
<tr>
<th>Disruptant</th>
<th>α-Amylase activity (U/ml)</th>
</tr>
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<tbody>
<tr>
<td>M-2-3</td>
<td>54</td>
</tr>
<tr>
<td>ΔamyR1</td>
<td>0.4</td>
</tr>
<tr>
<td>ΔamyR3</td>
<td>0.4</td>
</tr>
<tr>
<td>ΔamyR4</td>
<td>0.2</td>
</tr>
<tr>
<td>ΔamyR5</td>
<td>57</td>
</tr>
</tbody>
</table>

α-Amylase activity of each strain was measured as described in Materials and Methods.

report that describes the successful cloning of a transcriptional activator gene by anti-titration of the limiting transcription factor.

We previously reported that the transcription of the *amyB* and *glaA* genes was decreased significantly by the introduction of a number of improved promoter that contains multiple copies of region III and CCAAT box.† These sequences are conserved in the promoter regions of, and are required for efficient expression of the amylolytic genes, and this phenomenon was therefore assumed to be caused by titration of the common regulatory factor(s). Recently, CCAAT-binding factors of *A. nidulans* have been found to bind to the *amyB* (luxG2) and *amdS* promoters, and were called AnCP (*A. nidulans* CCAAT protein) and AnCF (*A. nidulans* CCAAT factor), respectively. Furthermore, AnCP is identical to AnCF and has been shown to be a heteromeric protein nearly related to the *S. cerevisiae* HAP complex.‡ This indicated the possibility that the reduced expression of the amylolytic genes is due to the titration of the limited amount of the AnCP/AnCF. However, a similar titration phenomenon was observed when multiple copies of the region III and CCAAT were introduced into *A. nidulans* harboring

**Fig. 6.** Growth of the *amyR* Disruptants on the Starch-containing Medium.

Three *amyR* disruptants, ΔamyR1, ΔamyR3, and ΔamyR4, and non-disruptant, ΔamyR5, were grown on the MM containing starch + glucose (left) or starch (right) as carbon sources at 30°C for 3 days.
Fig. 7. Northern Blot Analysis of the amyR Transcription in A. oryzae.

Total RNA was isolated from the mycelium grown on either glucose (G) or maltose (M) as a carbon source. Fifteen μg of total RNA was electrophoresed on an agarose/formaldehyde gel, blotted onto a Hybond-N membrane, and then hybridized with either 32P-labeled amyR (left) or agdA (right) as a probe. A very faint signal (indicated by an arrowhead) corresponding to the transcript of the agdA gene is found at almost the same position as 28S rRNA only in the RNA prepared from the maltose-grown mycelium. A photograph of the ethidium bromide-stained gel is shown at the bottom. Molecular sizes and the position of rRNAs are indicated in the panel.

The fusion gene of the amyB promoter lacking a typical CCAAT sequence and lacZ. In addition, the transformants could grow indistinguishably from the recipient strain on a medium containing acetamide or acrylamide as a sole nitrogen source (data not shown), indicating that intrinsic amds gene expression of the transformants would not be affected much and therefore titration of the CCAAT-binding factor, AnCP, might not occur.

Gene disruptants of the amyR had limited growth on the starch-containing medium and a significantly reduced productivity of the amylolytic enzymes (α-amylase, glucoamylase, and α-glucosidase). This indicates that the amyR gene is actually responsible for efficient and starch/maltose inducible expression of the Aspergillus amylolytic genes.

In comparison with the homologies among the zinc binuclear motifs of various fungal regulatory factors, AmyRp has the highest similarity with the S. cerevisiae maltose-regulatory factor, Mal63p. This high similarity in the zinc finger motif suggested that the target binding sequence of AmyRp could be similar to that of Mal63p. To date, the binding consensus sequence for Mal63p has not been clearly identified. It was first indicated that the sequence GAAAA(A/T) TTTGCCG, found in the intergenic region of the MAL61 and MAL62, was required for Mal63p binding.44,45 Further experiment using a DNase-I footprinting assay found several protected sites in the MAL61-MAL62 promoter and the MAL63 promoter itself.46 All of the sequences in the binding sites were very similar, i.e., CGGN6CGG, CGGN6CGC, or CGGN6CGG, where the intermediary N6 sequence is very AT rich. Such similar sequences have also been found in the region III conserved in the three A. oryzae amylolytic gene promoters; CGGN4(=AAAT- TTAAC)GGG in the amyB, CGGN4(=AAAT- TTAAC)CTG in the glaA, and CGGN4(=GCAAT- TTATC)GGG in the agdA. The high similarity of the sequences could suggest that the region III might be a recognition site or a binding site of the zinc finger protein AmyRp. Further experiments including gel mobility shift and DNase-I footprinting assay must be done to identify the binding sequence of AmyRp.

In addition to the zinc finger, AmyRp has a leucine zipper-like motif in the middle part of the protein, thereby it might bind to DNA as a homodimer. The target site of the zinc finger proteins that bind as dimers to DNA generally consists of a palindromic triplet sequence at each end of the site; for example, the binding site of the Gal4p, Ppr1p, and Put3p contains a 5′-CGG-3′ triplet at each end.47 However, as the predicted target site of AmyRp does not contain such a symmetrical sequence, it is not clear that the putative leucine zipper motif confers to yield a conformation with binding activity or AmyRp binds to the predicted target sites. Therefore, the role of the putative leucine zipper motif in the binding ability of AmyRp must be identified.

The transcriptional activator gene, amyR, and the amylolytic genes, agdA and amyA, are clustered on a 12-kb DNA fragment of the largest chromosome in A. oryzae, and the amyR and agdA genes are transcribed divergently from a 1.5-kb intergenic region. It has been known that the putative regulatory gene and the gene regulated directly by the regulator are divergently transcribed and share an intergenic region in Aspergillus. For example, alcR, the activator gene involved in the regulation of the ethanol-use regulon is transcribed in the opposite direction from the alcO gene, expression of which is controlled by alcR. Similar patterns of regulation of alcR and alcO, even though different in their expression levels, have indicated that they share a common cis-acting element for their regulation.48 In contrast, the amyR gene was expressed even in the presence of glucose comparable to the level in the presence of maltose, while the agdA gene was induced only by maltose. This result suggested that these genes do not share a common cis-element in the intergenic region and the expression of the amyR is not autoregulatory. The region III conserved in the amylolytic genes is about 400 bp upstream of the translation start codon of the agdA, but exists about 1100 bp apart from that of the amyR. Therefore, it is taken for granted that the
region III does not function as a cis-acting element for amyr expression. The existence of the proximal CT-rich stretch may contribute to a high constitutive level of expression of the amyr. amyr is constitutively transcribed but responsible for increasing the transcription efficiency of the amylolytic genes only in the presence of maltose or starch. One possible explanation is that the regulation at the translational level may occur, as is known in the case of the general amino acid control in yeast. In such case the transcriptional activator gene, GCNA, has upstream open reading frames (uORFs) that function as a translational regulator.25,26 Indeed, three uORFs are found upstream of the translation start codon in the amyr.

Very recently, however, a DNA-binding protein interacted with the region III sequence has been found in the nuclear extract of A. nidulans grown in the presence of glycerol, a non-inducer for amylolytic enzymes.27 This suggests that AmyRp is translated constitutively also in A. oryzae, but is inactive for transcription in the presence of glucose or glycerol. In the presence of an inducer (maltose), the structure of AmyRp would be altered to activate transcription by an unknown mechanism. It was reported that the C-terminal region of Mal63p comprises a maltose-responsive negative regulatory domain and probably interacts with the transactivation domain to inhibit the transcription activation.28 Maltose-induced conformational change would occur in Mal63p to activate transcription in the presence of maltose. This model could be applicable to the AmyRp activation mechanism, although detailed molecular analysis is required.

In this study, we demonstrated that multiple copies of the introduced amyr gene reversed the reduced expression level of the amyB promoter-controlled lacZ gene in the transformant containing a large number of region III sequence. It would be expected, therefore, that the expression of the gene of interest under the control of the improved promoter carrying multiple copies of region III can be increased further by introduction of the amyr gene in a multi-copy. This approach is underway to improve the protein productivity in our strong expression system.

Acknowledgments

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43) Steidl, S., Papagiannopoulos, P., Litizzka, O., Andrianopoulos, A., Davis, M. A., Brakhage, A. A., and Hynes, M. J., AnCF, the CCAAT binding complex of Aspergillus nidulans, contains products of the
A Transcriptional Activator Gene, amyR, of Aspergillus oryzae


