Overexpression of nreB, a New GATA Factor-encoding Gene of *Penicillium chrysogenum*, Leads to Repression of the Nitrate Assimilatory Gene Cluster*

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To investigate the mechanism of nitrogen metabolite repression in the biotechnologically important fungus *Penicillium chrysogenum* a polymerase chain reaction approach was employed to identify transcription factors involved in this regulatory circuit, leading to the isolation of a new gene (nreB) encoding a 298 amino acid protein. Despite a low overall amino acid sequence identity of approximately 30%, it shares several features with Dal80p/Uga43p and Gzf3p/Nil2p, both repressors in nitrogen metabolism in *Saccharomyces cerevisiae*. All three proteins contain an N-terminal GATA-type zinc finger motif, displaying 86% amino acid sequence identity, and a putative leucine zipper motif in the C terminal. Northern blot analysis revealed the presence of two nreB transcripts, 1.8 and 1.5 kilobases in length, that differ in polyadenylation sites. The steady state level of both transcripts is subject to nitrogen metabolite repression. The putative DNA binding domain of NREB, expressed as a fusion protein in *Escherichia coli*, binds in vitro to GATA sites of its own 5′-upstream region as well as in the promoter of the nitrate assimilation gene cluster. Consistent with a role in the regulation of nitrogen metabolism, overexpression of nreB leads to repression of nitrate assimilatory genes. Hence, the simple view of nitrogen regulation by four GATA factors in yeast, but only one key regulator in filamentous ascomycetes seems no longer valid.

Nitrogen metabolite repression controls the synthesis of a range of enzymes required for utilization of nitrogen sources in such a way that primary nitrogen sources, like ammonia or glutamine, are utilized preferentially (1–3).

Several structural as well as regulatory genes involved in this process have been isolated and characterized in *Saccharomyces cerevisiae* (1). To date, five global nitrogen regulatory factors are known to participate in the regulated transcription of nitrogen catabolic genes. Four of them, Gln3p, Dal80p (also termed Uga43p), Nil1p (also termed Gat1p), and Gzf3p (also termed Nil2p), are DNA-binding proteins that contain a single GATA-type zinc finger, recognizing the consensus motif GATA (4–9). Gln3p and Nil1p are transcriptional activators whereas Dal80p and Gzf3p (proposed to repress Gln3p- and Nil1p-mediated transcription possibly by competing for some binding sites) act as negative regulators of multiple nitrogen catabolic genes. The fifth factor, Ure2p, is considered to be a negative regulator of Gln3p via direct binding (10).

Most of our understanding of regulatory circuits in multicellular fungi is based on *Aspergillus nidulans* and *Neurospora crassa*. In these species a number of genes have been identified as having an influence on the nitrogen regulatory network directly or indirectly (2, 3). In contrast to *S. cerevisiae*, only one GATA factor has been implicated in nitrogen metabolite repression in both fungi at present: AREA from *A. nidulans* and NIT2, its homologue from *N. crassa*. Both proteins are highly related positive acting wide domain regulators (11, 12). The activity of NIT2 is modulated by the negative acting factor NMR through direct protein-protein interaction in *N. crassa* (13). Employing electrophoretic mobility shift assay analysis, at least three GATA binding activities other than AREA have been distinguished in *A. nidulans* (14). This suggests the existence of additional GATA factors possibly involved in nitrogen metabolite repression, thus resembling the situation in *S. cerevisiae*. However, a number of possibilities other than nitrogen regulation may pertain since fungal GATA factors have also been shown to be involved in regulatory circuits as different as iron metabolism in *Ustilago maydis*, mating type switching in *S. cerevisiae*, blue-light signal transduction, and circadian rhythmicity in *N. crassa* (15–18). Recently, a second GATA factor-encoding gene from *A. nidulans* has been described that appears to play a role in sexual development (19).

Hitherto, the knowledge about nitrogen control mechanisms in other fungi is rather fragmentary. The filamentous fungus *Penicillium chrysogenum* is the most important industrial producer of the β-lactam antibiotic penicillin. The synthesis of this secondary metabolite is assumed to involve nitrogen metabolite repression (20, 21), among other regulatory circuits. Recently, we have isolated the *areA/nit2* homologue nre from *P. chrysogenum* and demonstrated, besides its AREA/NIT2 homologous function, specific binding of an NRE fusion protein to double GATA elements in the promoter regions of the nitrate assimilation and penicillin biosynthesis gene clusters (22, 23).

The aim of the present work was to identify and characterize additional GATA factors involved in nitrogen control in filamentous fungi. To further our understanding of this complex regulatory network, we have cloned and characterized the gene encoding NREB, a new DNA binding GATA factor from *P. chrysogenum* that is able to act as a repressor of nitrogen catabolic genes.

**EXPERIMENTAL PROCEDURES**

Strains, Vectors, and Growth Media—The *P. chrysogenum* strain Q176, provided by the Biochemie GmbH (Kundl, Austria), was used throughout this study. All liquid cultures were inoculated with 10°

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U96365.

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conidiospores at 25 °C in 500-ml Erlenmeyer flasks on a rotary shaker (250 rpm) in 250 ml of minimal medium containing 30 mM NaNO₃, 7 mM KCl, 2 mM MgSO₄·7H₂O, 0.3 mM FeSO₄·7H₂O, 25 mM potassium phosphate buffer (pH 6.0), 2% sucrose, and trace elements according to Vogel. For Northern analysis, nitrate was replaced by the respective nitrogen source for regulation of the cytochrome c oxidase promoter (pY113) in the E. coli DH15a, which was used as plasmid vector and host, respectively.

Cloning of nreB—Fungal DNA and RNA were isolated according to Eytlon (24) and using RNaZol™ (Biotex Laboratories, Inc.), respectively. A 56-bp fragment of the zinc finger-encoding region of different fungal NREB factors was amplified utilizing two degenerate oligonucleotide primers, 5′-ACNCCNNTYTGTGGYM (Y = T or C; M = A or C; N = any nt; P = A or G) derived from the amino acid sequence Thr-Pro-Leu-Trp-Arg and 5′-APNCCPANGCPTTPCA, antisense according to the amino acid sequence Cys-Asn-Ala-Cys-Gly-Leu. In an alternative approach, GATA-factor-encoding cDNA was amplified according to the RACE protocol of Frohman et al. (25) as described previously (26). The amplification products were gel purified and subcloned into pGEM-T vector (Promega).

For determination of the 5′-end of the nreB cDNA, total Penicillium RNA was reverse transcribed using Superscript reverse transcriptase (Life Technologies, Inc.) according to the manufacturer’s directions and employing the oligonucleotide 5′-AGTCTCTGACACATC (corresponding to nt 523 in Fig. 1) as primer. Subsequently, the amplified product was d(A) tailed and amplified by PCR using the (dT)₁₇-adapter primer, the primer with Klenow fragment of DNA polymerase I using [α-3²P]dCTP (27).

NREB binding reactions were carried out in 20 μl of 12 mM HEPES-NaOH (pH 7.9), 4 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM EDTA, 1 mM dithiorthreitol, 2 μg of poly(dI-dC), and 10% glycerol, using 20,000 cpm of the respective 5′-end-labeled DNA fragment and different amounts of MBP::NREB. The reaction mixtures were incubated at room temperature for 20 min and subsequently loaded onto 4% polyacrylamide gels in 0.25× Tris-borate buffer (27). After electrophoresis, gels were dried and autoradiographed. Quantification of the intensity of shifted bands in comparison with the respective free probes was performed using a Phosphor Storage Imaging System, model Storm 840 (Molecular Dynamics).

For determining contact analysis, the end-labeled DNA fragments were partially depurinated prior to gel band mobility shift analysis. After separation, free DNA and DNA-protein complexes were isolated, cleaved at purine residues, and analyzed by electrophoresis in a sequencing gel and autoradiography as described previously (23).

Gene Disruption and Overexpression of nreB—A xyl1::pNREB fusion gene was generated by PCR-mediated ligation (31). The 1.6-kb upstream region of the xylose promoter fragment was amplified by PCR from a subcloned SalI fragment (20) employing the M13 reverse sequencing primer and the oligonucleotide 5′-GAAATATGCTAACTGAAGG for the 3′-downstream region of the xylose gene was used as the selection marker (32). Protoplasts were transformed according to Cantor et al. (33), and transformants were selected on minimal medium containing 10 μg/ml phleomycin (Sigma). Screening of positive clones was performed by PCR. To obtain homokaryotic transformants, colonies from single homokaryotic spores were picked, and genomic integration of the expression construct was verified by PCR and Southern analysis. Three different transformants carrying a single copy of pXNREB integrated ectopically were selected for growth tests and Northern analysis.

For disruption of nreB, a 1.9-kb HindIII-Clal fragment carrying the Trn5-phophoeycin resistance gene under the control of the isopenicillin-N-synthase promoter isolated from P. chrysogenum was used as the selection marker (32). Prototrophs were transformed according to Southern analysis. The three different transformants carrying a single copy of pXNREB integrated ectopically were selected for growth tests and Northern analysis.

RESULTS AND DISCUSSION

Isolation and Characterization of nreB—The various members of the GATA protein family are related by a high degree of similarity within their DNA binding domain. To isolate additional GATA factors from Penicillium, two different PCR-aided strategies that used degenerate oligonucleotide primers derived from two regions conserved in most fungal GATA factors were applied. In the first approach, cDNA was used as a template to amplify the 3′ part of the GATA factor-encoding transcripts as described previously (26) according to the RACE

1 The abbreviations used are: bp, base pair(s); nt, nucleotide; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; kb, kilobase pair(s).
protocol of Frohman et al. (25). In the second approach, genomic DNA was used as a template for conventional PCR to amplify a 56-bp fragment encoding the zinc finger region of the GATA factors. Both strategies yielded fragments of three different GATA factor-encoding genes: first, the already characterized major nitrogen regulatory gene \textit{nre}; second, \textit{sreP}, a putative homologue of the siderophore regulatory gene \textit{urbs1} from the basidiomycete \textit{U. maydis} (26); and third, a new gene, designated \textit{nreB}, encoding a GATA-type zinc finger motif similar to that of Dal80p and Gzf3p from \textit{S. cerevisiae}. To characterize the complete gene, including the adjacent non-coding regions, a 12-kb chromosomal clone was isolated from a \textit{EMBL3A} \textit{P. chrysogenum} phage library using the amplified cDNA clone as probe and subcloned into plasmid vectors. Subsequently, a 7.3-kb \textit{Sal}I-\textit{EcoRI} fragment containing \textit{nreB} was sequenced in its entirety (Fig. 1).

Comparison of the genomic and cDNA sequences revealed an open reading frame of 894 bp interrupted by a single 102-bp intron, 3' of the zinc finger-encoding region. The consensus sequences for the 5' and 3'-borders of the intron, GTPAY, and CTPAY, the putative internal consensus sequence for lariat formation, are conserved (34).

The deduced \textit{P. chrysogenum} NREB protein displays a calculated molecular mass of 32.7 kDa. Searches in several databases using the BLAST alignment computer program (35) confirmed that the cloned gene codes for a putative member of the NREB, a GATA Factor Involved in Nitrogen Regulation.
GATA family. NREB exhibits the strongest similarity to Dal80p and Gzf3p from S. cerevisiae (6, 8). The completion of the budding yeast genome sequencing project has made it possible to determine the exact number of genes of a particular type. In budding yeast, four CX_2CX_1_2CX_2-C-type GATA factors (Gln3p, Dal80p, Nil1p, and Gzf3p) have been characterized, and all four participate in nitrogen regulation. Alignment of the DNA binding motifs indicates 86% amino acid identity of NREB to both Dal80p and Gzf3p, 76% to NRE (Fig. 2), but only 56% and 64% to the two GATA-type zinc fingers of SREP (data not shown). In addition, sequence similarity of NREB, Dal80p, and Gzf3p is not exclusively limited to the zinc finger region. All three proteins display a putative leucine zipper motif in the C terminus (Fig. 2). In Penicillium NREB, the heptad pattern of hydrophobic amino acids is even longer and includes 11 residues (Fig. 1) in a region predicted to form a coiled coil structure by computer analysis using the ExPASy tools software package (36). Although not all of these residues are leucines, mutational analysis of the GCN4 leucine zipper has proved that both aromatic and aliphatic residues can functionally replace leucine residues without affecting the dimerization properties of this motif (37). In contrast to NRE and all other identified positive acting GATA factors involved in nitrogen regulation in fungi, NREB lacks regions rich in acidic residues putatively involved in transcriptional activation and in this respect also resembles Dal80p and Gzf3p. This is in accordance with the proposed role of NREB as an inhibitor of transcription.

Several additional motifs have been identified in the amino acid sequence of NREB. Among them are three putative nuclear targetting sequences, a 4-residue pattern at amino acid position 72 and the bipartite consensus sequence at positions 221 and 235 (38). Furthermore, 19 putative phosphorylation sites, including 4 for cAMP and cGMP dependent protein kinases, 5 for protein kinase C, and 10 for casein kinase, are detectable in the sequence (39). The motif S(T)PXX, occurring at high frequencies in gene regulatory proteins, appears six times in NREB (40).

The obvious similarity of the nitrogen metabolite repression system in filamentous ascomycetes gave rise to speculation that nreB homologous genes may also exist in the genetic model organisms A. nidulans and N. crassa. Results of PCR amplification and subsequent sequence analysis of the obtained fragments support this assumption and prove that this gene is not unique to Penicillium (3). Applying different PCR approaches, besides NREB or SREP homologues, no other CX_2CX_1_2CX_2-C-type GATA factor-encoding genes could be amplified from Penicillium, Aspergillus, or Neurospora. Our results suggest that the basic mechanism of nitrogen regulation is conserved in S. cerevisiae and filamentous fungi. In both systems, positive and negative acting GATA factors are engaged, but in contrast to yeast, only two GATA factors appear to be involved in nitrogen regulation in filamentous fungi.

Northern Analysis and Transcript Mapping.—To examine if transcription of nreB is subject to nitrogen control, total RNA was isolated from mycelia grown in different nitrogen sources and probed with nreB. Subsequently, the blots were stripped and hybridized with the major nitrogen regulatory gene nre, the nitrate reductase-encoding gene niaD, and the γ-actin-encoding gene as a loading control (Fig. 3). In mycelia grown in the presence of various derepressing nitrogen sources, nitrate, urea, alanine, or hypoxanthine, two transcripts, 1.5 and 1.8 kb in length, were detectable. In contrast, cells grown in ammonia or glutamine exhibited low levels of nreB transcript.

To investigate the mode of induction of nreB expression, mycelia were grown in glutamine and subsequently transferred into medium containing nitrate as sole nitrogen source. nreB transcripts were detectable 15 min after transfer. Interestingly, the steady state level of nreB mRNA displayed maximal expression about 1 h after derepression as already demonstrated for the nre gene (29). In mycelia grown for 36 h in a derepressing nitrogen source, the 1.5- and 1.8-kb mRNA species appeared approximately equimolar, whereas in the phase of derepression (20 and 60 min after shift), the shorter transcript was about 3-fold enriched. Although the significance of this observation remains to be elucidated one can speculate that the two transcripts might possess different features due to their different 3'-ends for example in mRNA stability. In this respect it is interesting that Platt et al. (41) recently demonstrated that nitrogen metabolite signaling involving the 3'-untranslated region of areA transcripts, encoding the major nitrogen regulatory gene of A. nidulans.

In summary, our results suggest that expression of nreB is subject to nitrogen metabolite repression similar to that of the nitrogen regulatory gene nre and the nitrogen catabolic gene niaD. This expression pattern is reminiscent of that of yeast DAL80 (6). In contrast, transcription of GZF3 is only slightly reduced by repressing nitrogen sources (8).

To determine the nreB transcription start points and polyadenylation sites, 5'- and 3'-RACE protocols as well as primer extension experiments were carried out. The single transcription start point detected is located 13 nt upstream of the putative start codon (Fig. 1). The two polyadenylation sites found are located at nt 1320 and 1640. Hence, the length of the proposed mRNA corresponds well with data from Northern blot analysis that revealed two transcripts. To verify that the transcript heterogeneity depends on two different polyadenylation sites, Northern blots were also probed with a 2.4-kb fragment directly 5' of the nreB transcription start site and separately with a fragment from a region between the two determined polyadenylation sites (Fig. 3, lanes 11 and 10, respectively). The latter probe specifically detected the 1.8-kb transcript, confirming the 3'-extension of the 1.8-kb transcript. Consis-

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2 I. Zadra and H. Haas, unpublished results.
FIG. 3. Northern analysis of Penicillium nreB gene expression. After growth of P. chrysogenum Q176 for 36 h under nitrogen-repressed conditions (glutamine as sole nitrogen source), mycelial pads were washed and transferred to medium containing nitrate. RNA was isolated at time points of 0, 5, 15, 60, and 360 min corresponding to lanes 1, 2, 3, 4, and 5. RNA loaded in lane 6, 7, 8, and 9 was isolated from mycelia grown for 36 h in ammonia, urea, alanine, and hypoxanthine, respectively. Blots were hybridized with a radiolabeled fragment of the nreB coding region. Subsequently, blots were stripped and probed with the Penicillium nre and niaD genes. As control for loading and quality of RNA, blots were hybridized with actA, encoding the γ-actin gene of P. chrysogenum. Lanes 10 and 11 represent the same RNA as shown in lane 4, probed with fragments corresponding to the region 3′-downstream of the first polyadenylation site and the 5′-upstream region of nreB, respectively.

FIG. 4. Northern analysis of an nreB overexpressing Penicillium strain (Q NOM) in comparison with the wild type strain (Q176). A. P. chrysogenum Q176 and QNOM were grown for 36 h in glutamine and sucrose as nitrogen and carbon sources (Q1). Subsequently, mycelial pads were washed, transferred into media containing nitrate and sucrose (NS), glutamine and xylose (QX), or nitrate and xylose (NX), and grown for another hour. Total RNA was isolated from mycelia of all four growth conditions, separated on an agarose/formaldehyde gel, blotted onto membrane filter, and hybridized with niaD and actA. Sequentially, the blot was stripped and hybridized with niaD, nreB, and nre. As a loading control and internal standard, the blot was probed with actA. The induction of the xyp transcription was verified by hybridizing the blot with xyp. B, quantification of mRNA levels normalized with respect to actA levels, using a PhosphorImager. Bars represent mean values of two independent experiments; standard deviations did not exceed 20%. Q176, open bars; QNOM, black bars.

Steady state mRNA level analyses of several regulatory and structural genes of one xylP:nreB+ transformant (Q NOM) indicate that the growth inhibiting effect on secondary nitrogen sources is due to repression of genes encoding nitrogen catabolic enzymes (Fig. 4). In QNOM, transcript levels of nreB were about 3-fold higher than wild type when grown in sucrose and 7-fold higher when induced with xylose. niaD and niaA mRNA levels were found to be approximately five times reduced when grown with sucrose, and no transcription of these structural genes was detected when grown with xylose as the carbon source (Fig. 4). In contrast to the nitrate assimilatory genes, the transcript level of nre was increased in QNOM suggesting that this positive regulatory gene does not appear to be controlled by nreB.

Therefore, the repression of the nitrate assimilation gene cluster is not caused by down-regulation of the activator NRE. An explanation for increased nre transcript levels in QNOM might be the induced nitrogen starvation due to overexpression of nreB. Repression of the nitrogen catabolic genes niaD and niaA in QNOM prompts us to conclude that nreB disruption would lead to derepression of nitrogen regulated genes. This phenotype would match that of a Saccharomyces DAL80−mutant. On one hand, it is puzzling that Dal80p, Gzf3p, or even

3 G.A. Marzluf, personal communication.
both proteins that display similar features as NREB, are dispensable for growth (8). On the other hand, the putative lethality of an nreB mutation is consistent with the fact that neither in A. nidulans nor N. crassa was such a mutant identified despite extensive screening for regulatory mutants of nitrogen control in both species. The known derepressed phenotypes are a result of certain mutations in the major nitrogen regulator-encoding genes, areA of A. nidulans, meaB of A. nidulans, and nmr of N. crassa (12, 13, 42). These mutations cause derepression of nitrogen-controlled genes under conditions of nitrogen repression. In contrast, S. cerevisiae DAL80 strains display inducer independent expression of nitrogen-controlled genes under derepressed conditions (6). Since nreB transcription was found to be confined to growth on secondary nitrogen sources, a function similar to Dal80p could also be predicted for NREB. The specific role of this new GATA factor of filamentous fungi in nitrogen control and the putative lethality of the disruption of its encoding gene certainly needs further investigation. Therefore, the functional analysis of nreB homologues in A. nidulans and N. crassa, species which are more amenable to classical genetics and molecular techniques, might be helpful.

**NREB Binds to GATA Motifs Present in Its Own Promoter and in the Promoter of the Nitrate Assimilation Gene Cluster**—GATA factors are defined as DNA-binding proteins that recognize the core motif GATA. To investigate whether nreB in fact encodes a DNA-binding protein, an N-terminal 76 amino acid peptide containing the zinc finger domain was expressed as a fusion with the maltose-binding protein in E. coli and purified by affinity chromatography. Inspection of the nreB promoter region revealed the presence of five closely spaced GATAA motifs, all in head to tail orientation in the immediate vicinity of the transcription start point. DNA sequences containing two or more adjacent GATA copies are known primary targets for various GATA factors in vitro and in vivo (23, 43, 44). Therefore, a 300-bp fragment spanning these GATA sites was chosen to investigate the DNA binding capacity of MBP::NREB. Mobility shift analysis proved that this fusion protein binds to the 300-bp fragment with high affinity (data not shown) as well as to the NarI digestion products (130 and 170 bp in length) containing three and two GATAA motifs, respectively (Fig. 5). Considering that expression of nreB is sensitive to nitrogen metabolite repression, binding of NREB to its own promoter region indicates autogenous regulation as proposed for other GATA factor-encoding genes like areA or DAL80 (5, 45). Alternatively or additionally, these binding sites may also attract other GATA factors like NRE.

The affinity of NREB to a synthetic 20-bp fragment that contains the single 3′ GATA site of the 170-bp fragment and is surrounded by the same nucleotides is about 10-fold less compared with the native 170-bp fragment. The enhanced affinity for sequences containing two GATA core elements suggests cooperative binding as shown for NIT2 and also proposed for AREA and NRE (23, 43, 45). Interestingly, GATA-1, a GATA factor involved in regulation of erythroid cell specific genes of vertebrates, self-associates whereby the zinc finger region of the molecule is sufficient to mediate this interaction (46, 47). In vivo NREB might bind as a dimer formed via the putative leucine zipper as suggested for Dal80p since this potential dimerization structure is essential for the function in S. cerevisiae (5).

The specificity of the protein-DNA interaction was determined by purine missing contact analysis. The results indicate that all five GATA sites of the promoter are involved in binding by NREB. In accordance with binding specificities of the GATA factors NIT2 and NRE, purines required for interaction with the zinc finger are not exclusively confined to the GATA core sequences, crucial purines were also found in the 3′-flanking region of both GATA motifs of the 170 bp fragment (Fig. 6).

Since overexpression of nreB resulted in down-regulation of niaD and niiA transcription, the promoter region of the nitrate assimilation gene cluster was analyzed for potential NREB binding sites. Electrophoretic mobility shift analysis employing the MBP::NREB fusion protein demonstrated that NREB interacts with two fragments (F100 and F120) containing two and three GATA sites, respectively (Fig. 5). This, together with the repression of niaD and niiA gene expression in an nreB overexpressing strain, suggests that NREB acts directly at the level of transcription. Since NREB recognizes binding sites that have already been shown to bind NRE, the major positive acting GATA factor of *Penicillium*, it is tempting to assume that these two regulators compete for the same binding sites (23). Regarding the role of GATA factors in determining both transcriptional activation and repression, another intriguing possibility is that NREB functions as a corepressor interacting physically with NRE whereby this interaction could be medi-
ated through the zinc finger domain. Examples of GATA and non-GATA transcription factors interacting directly with GATA domains have been identified in other systems (46–48). In such a model, overproduction of NREB leads to repression of nitrogen metabolite regulated genes by disturbing the balance between NRE and NREB. Recently, a negative-acting factor from A. nidulans has been identified genetically that is involved in determining the functional specificity of AREA and is proposed to operate through direct interaction with the zinc finger domain of AREA (14, 49).

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