Cycloheximide (CYH) resistance in the yeast *Candida malta* is based on the inducible expression of genes encoding a variant of ribosomal protein L41-Q, with glutamine at position 56 instead of the proline found in normal L41. The promoter of *L41-Q2a*, one of the *L41-Q* gene alleles encoding L41-Q, has an element similar to the Gcn4p-responsive element of *Saccharomyces cerevisiae*. In a previous study, this element was shown to be essential for the induction of L41-Q by CYH. In the present study, a *C. maltosa* GCN4 homolog, C-GCN4, was cloned. It had a long 5'-leader region with three upstream open reading frames. Enhanced expression of the C-GCN4 reporter fusion gene upon the addition of 3-aminotriazole or by mutations in start codons of all three upstream open reading frames indicates that C-GCN4 expression is under translation repression as was seen with GCN4. The C-GCN4-depleted mutant was unable to grow in a nutrient medium containing CYH and did not express L41-Q genes. Recombinant C-Gcn4p bound to the consensus DNA element for Gcn4p, 5'-GGATGTGACTCAT-3', located upstream of *L41-Q2a*. Thus, C-Gcn4p, which likely functions in the general control of amino acid biosynthesis, is essential for the expression of *L41-Q* genes.

Cycloheximide (CYH) is an antibiotic that inhibits the peptide elongation reaction on eukaryotic ribosomes by binding specifically to the 60 S subunit. Numerous yeast species, including *S. cerevisiae*, are sensitive to low concentrations of CYH. However, some species are by nature resistant to CYH, and their resistance is based on the production of a variant ribosomal protein L41. CYH-sensitive yeasts have a conventional P-type L41 protein, with proline as amino acid residue 56, whereas CYH-resistant yeasts have a Q-type L41 protein, with glutamine at position 56 instead of the proline found in normal L41. The promoter of *L41-Q2a*, one of the *L41-Q* gene alleles encoding L41-Q, has an element similar to the Gcn4p-responsive element of *Saccharomyces cerevisiae*. In a previous study, this element was shown to be essential for the induction of L41-Q by CYH. In the present study, a *C. maltosa* GCN4 homolog, C-GCN4, was cloned. It had a long 5'-leader region with three upstream open reading frames. Enhanced expression of the C-GCN4 reporter fusion gene upon the addition of 3-aminotriazole or by mutations in start codons of all three upstream open reading frames indicates that C-GCN4 expression is under translation repression as was seen with GCN4. The C-GCN4-depleted mutant was unable to grow in a nutrient medium containing CYH and did not express L41-Q genes. Recombinant C-Gcn4p bound to the consensus DNA element for Gcn4p, 5'-GGATGTGACTCAT-3', located upstream of *L41-Q2a*. Thus, C-Gcn4p, which likely functions in the general control of amino acid biosynthesis, is essential for the expression of *L41-Q* genes.

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C-Gcn4p, which likely functions in the general control of amino acid biosynthesis, is essential for the expression of *L41-Q* genes.

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1. The abbreviations used are: CYH, cycloheximide; GCN4, Gcn4p-responsive element; uORF, upstream open reading frame; 3-AT, 3-aminotriazole (3-amino-1,4,5-triazole); GST, glutathione S-transferase; YPD, yeast extract/peptone/dextrose; RACE, rapid amplification of cDNA ends.
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various fungi and yeast: cpc-1 in Neurospora crassa (17), cpcA in Aspergillus niger (18), cpcA in Aspergillus nidulans (19), cpcPC1 in Cryptococcus parasitica (20), and CgGcn4 in Candida albicans (21).

Under non-starvation conditions, Gcn4p is produced at low levels due to the negative effects of four upstream open reading frames (uORFs) in the leader sequence of GCN4 mRNAs (10, 11, 22). Under amino acid starvation conditions, the production of Gcn4p is induced by overcoming the inhibitory effect of uORFs through the function of Gen2p kinase (22–25). In addition to this translational regulation, Gcn4p is controlled through various mechanisms that are not yet entirely clear. First, Gcn4p is degraded rapidly in a SCF<sup>b</sup> ubiquitin ligase-dependent manner in vivo. This degradation process requires the phosphorylation of specific residues in the Gcn4p activation domain and the cyclin-dependent protein kinases Pho85p and Srb10p. Amino acid starvation or CYH administration somehow represses phosphorylation of Gcn4p and extends its half-life (25, 27–29). Second, Cpc2p regulates the transcription activation function of Gcn4p in a negative manner via an unknown regulatory mechanism. Cpc2p does not affect Gen2p-mediated Gcn4p expression, the stability of Gcn4p, or the binding ability of Gcn4p to GCRE (25, 30). Third, glucose addition or UV irradiation transiently activates Gcn4p through the Ras/cAMP signaling pathway (25, 31). This activation depends on Gen2p function but apparently not on its phosphorylating activity for eIF2α. Fourth, GCN4 mRNA levels rise to some extent under amino acid starvation or nitrogen starvation conditions (26, 32), although transcriptional control seems to be of only minor importance in the regulation of Gen4p function (32). Finally, decay of GCN4 mRNA is suppressed by a stabilizer element located between uORF4 and the GCN4 protein-coding region. This stabilization is mediated by a specific binding factor, Pub1, which is one of the major polyadenylated mRNA-binding proteins (33).

In C. maltosa, a similar general control system seems to be at work because amino acid starvation induces the expression of a histidine synthetase gene, C-HHis5, which has a GCRE-like element in its promoter. The same starvation conditions also induce the expression of L41-Q2a. Conversely, CYH also induces C-HHis5 (5). Deletion of the GCRE-like element or mutations in its consensus nucleotide sequence abolishes CYH-induced transcription of the L41-Q2a promoter (5). These observations lead to the assumption that the same Gcn4p-dependent mechanism is involved in both the general control of amino acid biosynthesis and induction of L41-Q2a by CYH.

This paper describes the cloning and characterization of C-GCN4, which encodes a Gcn4p homolog in C. maltosa. C-GCN4 mRNA was increased by the addition of CYH or 3-amino-triazole (3-AT), which blocks histidine biosynthesis, indicating the presence of control at the level of transcription or mRNA decay. The presence of control at the level of transcription or mRNA decay is A, C, G, or T).

PCR Amplification of the Conserved Region of C-GCN4 and Its 5′-Extended Region

A part of a GCN4 homolog was amplified from C. maltosa genomic DNA using the following forward and reverse primers, respectively: 5′-CCWTCRACWCCWATHG-3′ and 5′-TTCNACYCTRCTTCTCHAR- TTG-3′ (where R is A or G; W is A or T; Y is C or T; H is A, C, or T; and N is A, C, G, or T). 5′-Rapid amplification of cDNA ends (RACE) was performed using a 5′-full RACE core set (Takara Shuzo, Kyoto, Japan) according to the manufacturer’s protocol. The 5′-phosphorylated reverse transcription primer (5′-GACTACCTTCTCACCGTTG-3′), A1 (5′-GCAACGAT- GATCTCAATTACCC-3′), S1 (5′-AGCTACTAGATACAGGCGCGG-3′), A2 (5′-ACAAAGTGGCCTGAGGCGG-3′), and S2 (5′-AGAGAGATCAT- GACTGCTGTA-3′) were used to generate an extended C-GCN4 PCR product. The resultant 250-bp PCR product was sequenced, and the 5′-extension of conserved C-GCN4 mRNA was confirmed.

Plasmids

Construction of C-GCN4 Deletion Cassettes—A 2.7-kb EcoRV fragment containing C-GCN4 was inserted into the Smal site of vector pUC18 to obtain pUC-CGCN4. As a result of this, the entire C-GCN4 open reading frame was removed by digestion with AhiI and BstNI. The residual fragment was blunt-ended and was combined with a blunt-ended Sall fragment containing C-ade1 (39) to obtain pUC-CGCN4I or with a blunt-ended Sall fragment containing C-his5 (40) to obtain pUC-CGCN4I.

Construction of pPL-CGCN4 with a C-GCN4-LAC4 Fusion Gene—A 1-kb fragment containing C-GCN4 promoter and its 5′-coding region was amplified using primers 5′-ACCACTGCGCACTGCACTTATGTTAGTATA-3′ (primer 1) (Sall site is underlined) and 5′-AGTATACACATTATCTCCTCCTTTGCTAC-3′ (primer 2) (modified). The amplified fragment was digested with Sall and inserted between the Sall and Smal sites of pPLI (41). The resultant plasmid pPL-CGCN4 carries C-ade1, C-en, ARS, and the C-GCN4-LAC4 fusion in which LAC4 is fused in-frame to the ninth codon of C-GCN4 (42). The correct nucleotide sequence was confirmed by sequencing.

Construction of C-GCN4-LAC4 Derivatives with Mutations at uORF Start Codons—First, six DNA fragments (uORF11, uORF12, uORF21, uORF22, uORF31, and uORF32) were amplified from pUC-CGCN4 as the case of m-uORF1 to m-uORF3. The amplified fragment was digested with Sall and inserted between the Sall and Smal sites of pPL-I (41).

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

CMT100 (ade1::ADE1, his5::HIS5, ura3::URA3), CMT101 (ade1, his5::HIS5, ura3::URA3), and CMT102 (ade1::ADE1, his5::HIS5, ura3) are derivatives of C. maltosa strain CHAU1 (ade1, his5, ura3) and were used as the control strain for varioustransformants. C. maltosa IAM12247 (the wild-type strain) has been described previously (3).

The expression of glutathione S-transferase (GST) fusion protein was induced in the strain Escherichia coli DH5 (supE44 hsdR1 recA1 endA1 gyrA96 thi-1 relA1).

The A-GCN4 strain was constructed by sequential two-step gene replacements of the chromosomal C-GCN4 genes using the PvuII fragment of the C-GCN4 deletion cassette (34, 35). Then the Ura<sup>+</sup> phenotype was rescued by transformation with a linear XhoI fragment containing the intact C-Ura3 gene from vector pURa3 (36). Isolation of total DNA from yeast cells was described in a previous study (37). Transformations were carried out using the lithium-acetate yeast transformation method (38). Correct replacement was confirmed by Southern blot analysis.

Yeast cells were cultivated in minimal or YPD medium at 30 °C. Appropriate supplements were added at the recommended amounts. Routine precultures were grown overnight, diluted with fresh media, and cultivated to an optical density of ~0.8 at 660 nm. Then, 50 μg/ml CYH or 10 μM 3-AT for histidine starvation was added to the culture. After an appropriate growth period, cultures were harvested for RNA extraction and measurement of β-galactosidase activity.

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produce PCR products m-uORF2 and m-uORF3, respectively. These DNA fragments were introduced between Sall and SmaI sites of pPL1 (41) as described above to give pPL-CGCN4m-uORF2 and pPL-CGCN4m-uORF3, respectively. The uORF2 start codon changes to CAA, and the uORF3 start codon changes to AGT.

A DNA fragment (uORF23) was amplified from m-uORF3 as a template using primers 6 and 2. uORF21 and uORF23 were digested with MnlI and Xhol from their ligation products, another uORF23 fragment as an (uORF13) was amplified using primers 4 and 2. SpeI-digested uORF11 and Xhol-digested uORF13 were ligated and, then, from their ligation products, a DNA fragment (m-uORF1,2,3) was amplified using primers 1 and 2. The fragment m-uORF1,2,3 was digested with Sall and inserted between the Sall and SmaI sites of pPL1 (39) to give pPL-CGCN4m-uORF1,2,3. The start codons of uORF1, uORF2, and uORF3 on m-uORF1,2,3 were changed to AGA, CAA, and AGT, respectively.

Nucleotide Sequence Determination

The nucleotide sequences were determined by the dideoxy chain termination method using the Applied Biosystems sequencing system model 310 DNA sequencer (Applied Biosystems, Foster City, CA).

Deletion Mutants of the L11-Q2a Promoter Region—All the deletion mutants of the L11-Q2a promoter region have been described previously (5).

Electrophoretic Mobility Shift Assay of DNA Binding

β-Galactosidase activity of permeabilized yeast cells were determined using ortho-nitrophenyl-β-D-galactopyranoside as a substrate. Yeast cells were cultivated in minimal medium, and β-galactosidase activities after the addition of CytH or 3-AT were assayed as described previously (41).

Disruption of C-GCN4 Simultaneously Abolishes the Inducible Resistance to CYH and the General Control Response in C. maltosa—To determine the function of C-Gcn4p in inducible resistance to CYH, two C-GCN4 alleles of the strain CHAU1 (ade1, his5, ura3) were disrupted successively using two disruption cassettes, one with C-HIS5 and the other with C-ADE1 as selective markers, and the results were confirmed by Southern analysis. Because direct disruption of the second C-GCN4
allele was unsuccessful, the second disruption was done in the presence of a centromere plasmid carrying wild-type C-GCN4, which was later transformed. The resultant Δc-gcn4 disruptor was then transformed with URA3 to avoid the influence of ura3 mutation and named ΔC-GCN4.

The ΔC-GCN4 strain grew as well as the wild-type strain in YPD medium; however, it grew slowly and formed pseudohyphae in minimal medium (45). This phenotype is in contrast to that of a C. albicans CaGcn4-disrupted mutant that did not show pseudohyphal growth under amino acid starvation (21). These results indicate that C-GCN4 is not essential for C. maltosa but is required for better growth of this yeast in minimal medium. In the presence of 50 μg/ml CYH, the ΔC-GCN4 strain failed to grow in YPD (Fig. 2A) or in liquid minimal medium (Fig. 2B) and was no longer inducible in resistance to CYH. These results indicate that C-Gcn4p is essential for the induction of CYH resistance in C. maltosa.

In C. maltosa, expression of L41-Q genes is required for resistance to CYH (3). Fig. 3, A and B, shows that neither CYH treatment nor histidine starvation induced the expression of L41-Q in the mutant ΔC-GCN4 in contrast to the case in the wild-type strain CMT100. The same conditions induced expression of C-HIS5 in ΔC-GCN4 but at a far lower level than in CMT100. These results indicate that C. maltosa C-GCN4 has a critical role in the transcriptional induction of both L41-Q and C-HIS5 genes under either CYH treatment or histidine starvation conditions.

Specific Binding of C-Gcn4p to the GCRE-like Element of L41-Q2α—To investigate the DNA region bound by C-Gcn4p, C-Gcn4p combined with glutathione S-transferase at its N terminus was produced in E. coli and affinity-purified using a glutathione-Sepharose 4B (Amersham Biosciences) column. The purified fusion protein showed the expected mobility when subjected to SDS-PAGE (Fig. 4A). In the electrophoretic mobility shift assay in which the purified protein was employed, the intensity of shift bands of either a 39-bp synthetic DNA fragment containing both the GCRE-like element and the GT-rich region (Fig. 4G) or a 21-bp DNA fragment containing only the GCRE-like element increased in proportion to the amount of purified recombinant fusion protein (Fig. 4, B and C). However, shift bands of a 24-bp DNA of only the GT-rich region were not observed (Fig. 4D). The shift band intensity of the 39-bp fragment was reduced along with the amount of unlabeled DNA fragments containing both the GCRE-like element and the GT-rich region or containing only the GCRE-like element (Fig. 4, E and F). Scintillation counting confirmed that the specific radioactivity of the 32P-labeled DNA probe containing both the GCRE-like element and the GT-rich region was almost equal to that containing only the GCRE-like element. Therefore, the binding of C-Gcn4p to the fragment containing both the GCRE-like element and the GT-rich region was ~2-fold higher than that to the fragment containing only the GCRE-like element (Fig. 4, B and C). These results indicate that C-Gcn4p binds specifically to the GCRE-like element of the L41-Q2α promoter and binds more favorably to the
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This paper presents several lines of evidence in favor of transcription activation by a Gen4p homolog through binding to a GCRE-like element of the L41-Q2a promoter as a mechanism of inducible resistance to CYH in C. maltosa (a schematic overview is given in Fig. 5). The experimental data supporting this notion are as follows: 1) disruption of C-GCN4 severely inhibited CYH-induced expression of L41-Q genes (Fig. 3A); 2) over-expression of C-GCN4 led to increased expression of L41-Q genes and earlier induction of CYH resistance (data not shown); 3) C-Gcn4p bound specifically to the GCRE-like element in vitro in the electrophoretic mobility shift assay (Figs. 4B–F); and 4) deletion of the GCRE-like element from the L41-Q2a promoter abolished the CYH-induced expression of a reporter gene (Fig. 4G) (5). Genes encoding ordinary ribosomal proteins are repressed in response to various starvation or stress conditions (46–49). Recent microarray analysis of Gen4p-dependent gene regulation in S. cerevisiae showed that 90 ribosomal protein genes were repressed by 3-AT (50). This paper therefore describes an interesting case in which a gene encoding a ribosomal protein gene was induced by CYH or 3-AT through regulation of a GCN4 homolog.

Genetic and molecular studies of the general amino acid control transcription activator Gen4p of S. cerevisiae have revealed specific functional domains that include a transcription activation domain, a basic DNA binding domain, and a leucine zipper-based dimerization domain (15, 16). C-Gen4p has similar structural features and should have similar functions to those of Gen4p. In fact, recombinant C-Gen4p bound to the AP-1 consensus sequence (5'-G/A/G/A/TGACCTCAAT-3'), as observed with Gen4p and other homologs. Furthermore, C-Gen4p was required for the induction of C-HIS5 mRNA under histidine starvation conditions (Fig. 3B), demonstrating the operation of a C-Gen4p-mediated general amino acid control pathway in C. maltosa. Thus, it is now obvious that C-Gen4p functions as a transcription activator both in the general control of amino acid synthetic genes and in the induction of L41-Q genes, which are responsible for CYH-resistance in C. maltosa. In S. cerevisiae, cellular Gen4p is maintained at a low level unless it is needed and Gen4p is phosphorylated by Pho85p and rapidly degraded in a SCFcdc ubiquitin ligase-dependent manner (28). Therefore, it is interesting to see how C-Gen4p is maintained or activated after the addition of CYH.

S. cerevisiae Gen4p homologs have a sequence similarity to c-Jun transcription factor, and like c-Jun, they bind to the AP-1 consensus response element. Gen4p has been shown to have functional similarity to c-Jun (51). In mammalian cells, treatment with protein synthesis inhibitors such as CYH or anisomycin accentuates and prolongs the induction of immediate early genes, including c-jun, in response to polypeptide growth factors and phorbol esters (52–56). Although the mechanism of this response is not clear, this process is thought to result from a combination of mRNA stabilization, activation of intracellular signaling cascades, and interference with transcriptional down-regulation (52–56). If the effect of CYH treatment on C. maltosa C-GCN4 is similar to that on c-jun in mammalian cells, a similar activation mechanism might be working. CYH binds to the 60 S ribosomal subunit, its only known target, producing translation arrest and polysome stabilization, which might activate an as yet unknown intracellular signaling system and result in stabilization of mRNA or even stabilization of

one accompanied by the GT-rich region. The observed C-Gen4p binding properties to various GCRE-like regions are consistent with the promoter activities as shown in Fig. 4G. In a previous paper (5), the promoter containing the GCRE-like element without the GT-rich region was reported to be not active in the induction of L41-Q2a. In this study, however, we found that it was able to induce L41-Q2a expression to some extent after the addition of CYH (Fig. 4G, see the case of pPLGCRE). These results indicate that binding of C-Gcn4p to the GCRE-like element is essential for the induction of L41-Q2a expression and that the GT-rich region is necessary for full induction of L41-Q2a by helping C-Gcn4p to bind to the GCRE-like element.

**DISCUSSION**

**Fig. 2.** Effect of CYH on the growth of the ΔC-GCN4 strain. A, growth on YPD agar medium with (top) or without (bottom) 50 μg/ml CYH. Strains were grown at 30 °C for 2 days. B, growth in liquid minimal medium with (filled symbols) or without (open symbols) 50 μg/ml CYH. CYH was added at about 0.08 OD660 (indicated with an arrow). Culture turbidity was automatically monitored with Biophotorecorder (model TN-112D, Toyo Co., Tokyo, Japan). Square, strain CMT100; circle, strain ΔC-GCN4.

**Fig. 3.** Effects of C-GCN4 disruption on L41-Q and C-HIS5 mRNA levels after the addition of CYH or 3-AT. Total RNA was prepared from culture samples of strain CMT100 or ΔC-GCN4 after addition of 50 μg/ml CYH (A) or 10 mM 3-AT (B).

- **A**
  - Wild-type
  - ΔC-GCN4
  - C-HIS5
  - L41-Qs
  - ACT1
- **B**
  - Wild-type
  - ΔC-GCN4
  - C-HIS5
  - L41-Qs
  - ACT1
C-Gcn4p. This signaling system, if any, might not be the same one as that resulting from derepression of C-Gcn4p synthesis upon amino acid limitation because disruption of two alleles of a gene encoding a Gcn2p homolog in *C. maltosa* made this yeast sensitive to 3-AT but not to CYH.²

Although the CYH treatment of 50 μg/ml severely inhibits protein synthesis of *C. maltosa* during an early stage of the CYH resistance induction, there remains some protein synthesis. In an experiment where 35S-labeled methionine and cysteine were taken up for 60 min after 3 h from the addition of CYH, the radioactivity in the trichloroacetic acid-insoluble ribosome fraction of the CYH-treated cells was not less than 2.5% of that of untreated cells (data not shown). This level of protein

² H. Takaku, E. Mutoh, Y. Sagehashi, R. Fukuda, H. Horiuchi, K. Ochi, M. Takagi, and A. Ohta, unpublished data.

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**Fig. 4.** DNA binding of the recombinant C-Gcn4p and the activities of L41-Q2a promoter elements. A, lane 1, crude extract of E. coli cells expressing GST-C-Gcn4p; lane 2, affinity-purified GST-C-Gcn4p. Proteins were separated by SDS-PAGE under the conditions described in the text. B–D, effects of GST-C-Gcn4p protein concentration on binding to DNA fragments containing the GCRE-like element and the GT-rich region (B), the GCRE-like element only (C), and the GT-rich region only (D). Protein concentrations in the reactions of lanes 1–7 were 20, 0, 1, 2, 5, 10, and 20 nM, respectively. E and F, competition in binding of GST-C-Gcn4p to the GCRE-like element and the GT-rich region by cold DNA fragments with the same sequence as the probe (E) or with only the GCRE-like element (F). 20 nM GST-C-Gcn4p was added to the respective reactions. The reactions of lanes 3–5 contained unlabeled DNA at concentrations of 1×, 10×, and 100× that of the labeled probe, respectively. G, various L41-Q2a 5′-promoter constructs were placed upstream of the β-galactosidase gene. Each number at the end of the left-hand bars indicates the position of the deletion end point from the start codon of the wild-type sequence (A of the ATG is designated as +1). Cells with respective constructs were grown for 6 h after the addition of 50 μg/ml CYH, and their β-galactosidase activities were measured. CYH was added when OD₆₆₀ reached 0.8. Values represent the average of at least three independent measurements.
synthesis will explain why L41-Q is induced under the high concentration of CYH. This leakiness of inhibition by CYH is probably brought about by another CYH resistance mechanism because a C. maltosa mutant that lacks all the functional L41-Q genes, L41-Q2a, L41-Q2b, and L41-Q3 (L41-Q3 has no allele), was able to slowly grow on 25 μg/ml CYH. This resistance is at least in part due to a homolog to C. albicans CaMDR1, which gave high CYH resistance to S. cerevisiae CaMDR1, because a mutant that lacks all the functional CaMDR1 gave high CYH resistance to S. cerevisiae.

This study focused on the identification and functional analysis of the upstream regulatory pathway in response to CYH or histidine starvation. Response to CYH or histidine starvation leads to the expression of C-GCN4. C-Gcn4p binds to the GCRE-like element of L41-Q promoter and induces the expression of L41-Q. Ribosomes with Q-type L41 proteins are then synthesized, and C. maltosa exhibits resistance to CYH.

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**FIG. 5.** Schematic view of transcriptional regulation of L41-Q genes. Response to CYH or histidine starvation leads to the expression of C-GCN4. C-Gcn4p binds to the GCRE-like element of L41-Q promoter and induces the expression of L41-Q. Ribosomes with Q-type L41 proteins are then synthesized, and C. maltosa exhibits resistance to CYH.
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