The Cysteine-Peptidase Bleomycin Hydrolase Is A Member of the Galactose Regulon in Yeast*

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Received for publication, June 4, 1997, and in revised form, August 26, 1997

Bleomycin hydrolase is a cysteine peptidase discovered through its ability to detoxify the anti-cancer glycopeptide, bleomycin. Although found in all tissues in mammals and in both eukaryotes and prokaryotes, the normal cellular function of this peptidase is not known. We had previously reported the purification of bleomycin hydrolase from yeast based on its unexpected ability to bind DNA. Recently we collaborated in solving the crystal structure of this protein, revealing a hexameric ring organization. We now report that the molecular characterization of the gene encoding yeast bleomycin hydrolase is also surprising. The transcription of the gene is regulated by galactose. Furthermore, this regulation is conveyed by a binding site for the Gal4 regulatory protein in its promoter, prompting the designation of this gene as GAL6. Gal6p also appears to have a negative effect on the GAL system as a deletion of the gene leads to a 2–5-fold higher expression of the GAL1, GAL2, GAL7, and MEL1 genes. The GAL6 deletion does not affect the expression of another inducible gene, HSP26. Neither the peptidase nor the nucleic acid binding activity of Gal6p as assayed is apparently required to convey this regulation, implying yet another function for this new member of the GAL regulon.

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The bleomycins are a family of glycopeptides produced by Streptomyces verticillus that can bind to and cleave DNA (1, 2). This property has been exploited widely for cancer chemotherapy (3). Most cell types express bleomycin hydrolase, an enzyme which renders bleomycin unable to cleave DNA and therefore nontoxic, whereas some tumors overexpress the enzyme (4). The level of expression of this hydrolase has been found to be low in yeast grown on glucose medium and induced severalfold on galactose medium. A Gal4p-responsive UASGAL was identified in the promoter region of the gene. It was shown to be responsible for this galactose regulation, prompting its designation as GAL6. Surprisingly, the deletion of the gene increases the expression level of galactose regulated genes, indicating that yeast bleomycin hydrolase, GAL6, is a negative regulator of the galactose regulon. This is the first evidence that a bleomycin hydrolase protein has a cellular function beyond detoxification of bleomycin.

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The Escherichia coli strain TG1 was used for plasmid amplification. Bacteria were grown in 2 × YT plus 25 μg/ml ampicillin. Saccharomyces cerevisiae strains used were Sc3163MATα αgal4-1 αgal80 GAL80::URA3 ura3-52 leu2-3, 112 his3 trp1-1 MEL1 GAL1/10::LacZ, Sc4574MATα αgal4 αgal80 αgal6::TRP1 GAL80::URA3 ura3-52 leu2-3, 112 his3 trp1-1 MEL1 GAL1/10::LacZ, diploid W303MATα/a ura3 ura3 leu2 leu2 his3 his3 trp1 trp1, haploid W303MATα ura3 leu2 his3 trp1, and Sc377MATα ura3 leu2 his3 trp1 αgal6::Trp1. Yeast were grown either in rich medium (YEP) or selective medium as necessary with appropriate carbon source. Carbon sources were sterilized separately and added to the medium to a final concentration of 2% glucose for the repressed condition, 3% glycerol plus 2% lactic acid, pH 5.7, or 2% raffinose for the induced condition, and 2% galactose for the induced condition.

**Protein Purification and Mass Spectrometry**—Yeast bleomycin hydrolase was purified from yeast cells grown in galactose as described previously (10). The molecular weight of purified protein was measured by mass spectrometry. Electrospray mass spectra were acquired with a VG QUATTRO II triple quadrupole Mass Spectrometer (Micromass Instruments, Manchester, UK) equipped with an electrospray interface at Howard Hughes Medical Institute Biopolymer Core Facility at the University of Texas-Southwestern.

**Plasmide**—The vectors pUC118 (14), YEP352 (15), pVT102u (16), and YIP56ER (17) have been described. The original GAL6 clone was obtained from a yeast genomic library in YEP24 and designated pXU2.

The primers 28cl (5'-CAACCCAGAACACGCGAGGCAGTCTG-3') from 780 bp upstream of the GAL6 ORF and Nsi (5'-AGCCGAGGAAA-

* This work was supported by National Institutes of Health Grants GM40700 and CA67982 and the Council for Tobacco Research-U. S. A. Grant 4247 (to S. A. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: UAS, upstream activating sequence; ORF, open reading frame; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); Tricine, N-[2-hydroxy-1,1-bis(hydroxy-

methyl)ethyl]glycine.
AGAACAGGGCTTATAGCAGAA-3') from 300 bp downstream of the GAL6 stop codon were used to amplify a PCR fragment that contains the GAL6 ORF and intact promoter from pXU2. This PCR fragment was cloned into the multiple cloning sites of YEP352 and designated pXU-606. The plasmid pXU616 is the same construct as pXU606 except that the cassette of the yeast HIS3 gene was replaced with the GAL6 promoter (UAS -galactosidase) as described previously (10).  

The SacI/BamHI fragment of the GAL6 gene from pXU606 was cloned into pUC118. A 1.8-kb BamHI fragment that contains the yeast HIS3 locus was cloned into the SacI-BamHI site of pUC118 modified by replacing the SacI-BamHI site with the HIS3 locus of Sc454. XhoI in the 3'-flanking sequence of GAL6 was used to introduce the EcoRI site in YIP356R. The GAL6 ORF is in-frame with the lacZ reporter gene in YIP356R. The resulting reporters were then integrated into the URA3 locus in the yeast strain Sc617 to study the regulation of GAL6 by galactose.  

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**RESULTS**

The Structure of the Gene Encoding GAL6—We previously reported the purification of a 48-kDa protein from yeast based on its DNA binding activity (10). We first obtained amino acid sequence from four tryptic digest fragments of this protein. Based on these sequences the corresponding gene was isolated and the predicted 48-kDa protein identified as the yeast homolog of the rabbit bleomycin hydrolase. Because we had shown that the levels of this protein were regulated by galactose, it was designated as GAL6 (see below).

The GAL6 ORF would encode a protein of 51.9 kDa if the +1 ATG designated in Fig. 1A is used. There is another upstream ATG that would encode a protein of 55 kDa if it were the authentic start site. This upstream ATG was designated as the start in previous publications (7, 8). The ATG at the downstream site matches the +1 ATG for the translational initiation sequence better (9/10) than the upstream start site (Fig. 1A) (9, 20).

To determine which ATG is used as the translation start site, we purified Gal6p from a wild type yeast strain and measured its molecular weight by mass spectrometry. As shown in Fig. 1B, the majority of purified protein has the molecular mass of 51.8 kDa, which is consistent with the use of downstream ATG.
as the translational start codon of GAL6. Our proposed translation of Gal6p would put its N terminus in alignment with that of the recently cloned human homolog (4, 21). The N terminus of Gal6p was not discernible in the crystal structure (22). Enenkel and Wolf (7), Magdolen et al. (8), and Pei and Sebti (23) assumed that the upstream ATG was the initiation site. We have now entered the corrected sequence into GenBank with accession number U74299.

GAL6 Is Regulated by Galactose—Transcription of the galactose genes in yeast is regulated by the transcription activator Gal4p. All the known galactose-regulated genes have a well characterized 17-bp Gal4p binding site (UASG) in their promoters. In glucose medium, the expression of GAL4 is suppressed, resulting in most, but not all, GAL genes being completely off. In glycerol medium, Gal4p levels are elevated, facilitating binding to the UASG. However, the negative regulator GAL80 suppresses GAL4 activity and blocks the expression of the GAL regulon (24). When cells are grown in galactose, Gal4p binds to the UASG of the galactose-regulated genes and activates transcription (13). By several criteria we find that GAL6 is also a member of the galactose regulon.

Gal6p was originally discovered in our lab by its ability to bind to DNA. It is the most significant activity in yeast cell extract that binds single-stranded DNA oligonucleotides in a gel mobility shift assay. We therefore used a gel shift as a sensitive assay to quantitate the amount of active Gal6p in cells expressing various amounts of Gal4p. As shown in Fig. 2A, the amount of Gal6p in the cell is proportional to the Gal4p levels. Deleting GAL4 (lanes 1 and 4) decreases the level of Gal6p, whereas increasing Gal4p levels with a multicopy plasmid increases Gal6p levels (lanes 3 and 6). As expected for a
Gal4p/galactose-regulated gene, when cells are grown in raffinose medium, Gal6p is expressed only at a low level. However, when cells are grown in galactose, the expression level of Gal6p increases approximately 5-fold over the glucose or glycerol levels (Fig. 2A, compare lanes 2 and 5). An immunoblot for Gal6p levels reveals essentially the same relative effects of galactose induction and Gal4p response as the gel shift (Fig. 2A, bottom). These differences in Gal6p protein levels are paralleled by the regulation of GAL6 mRNA levels, as shown by a Northern blot (Fig. 2B). This type of regulation is qualitatively the criteria for a Gal4p-regulated gene and quantitatively very similar to that of GAL80 and GAL3 genes (see “Discussion”).

The regulation of GAL6 mRNA levels implies a Gal4p-mediated control. Consistent with this deduction, we found a single Gal4p binding site (UASGal6) in the promoter region of the GAL6 gene (Fig. 1A). As shown in Fig. 2C, Gal4p can bind to the UASGal6 in vitro. To test whether the UASGal6 is responsible for the galactose regulation of GAL6 expression, we fused the GAL6 promoter, with or without the UASGal6, to a lacZ reporter gene. As shown in Fig. 2D, the β-galactosidase activity under the control of the wild type GAL6 promoter is regulated by galactose. A deletion of a 30-bp fragment containing the UASGal6 from the promoter eliminates this galactose regulation, indicating that the UASGal6 is responsible for the galactose regulation of GAL6 gene. The difference in reporter levels under repressing conditions (glucose) (60 versus 12 units) could be due to the 30-bp deletion disrupting binding of some other protein or that the Gal4-Gal80 complex contributes to the activation of GAL6 on glucose. Regardless, we conclude that GAL6 expression depends on Gal4p activity and that GAL6 is a newly identified member of the GAL regulon.

GAL6 Is a Negative Regulator of GAL Regulon Expression—Since GAL6 is regulated by galactose, we addressed the question of what role it might play in galactose regulation by deleting it. The deletion strain is viable with no notable effect on growth rate of glucose-grown cells (data not shown), which is consistent with previous observations (7–9). However, when we compared the induction of a galactose-regulated gene in the wild type and gal6 deletion strain, we found that GAL6 has a negative effect. In the gal6 deletion strain, the induction level of a β-galactosidase reporter under control of the GAL1/GAL10 promoter is higher than that in the wild type cells (Fig. 3A). The cells in this assay were transferred from repressing glucose medium to galactose medium at late mid-log phase, so they only grew a few generations before reaching stationary phase (final A600 nm ~ 4), accounting for the relatively low β-galactosidase activity. This assay was also performed for the enzyme Hsp26 (Fig. 3B). The a-galactosidase activity was 2.2-fold higher in the deletion strain (data not shown). This protocol emphasizes the effects of the kinetics of derepression from glucose in the GAL6 and Δgal6 strains. When the same GAL6 and Δgal6 strains were grown continuously in galactose medium there was also more β-galactosidase expression in the deletion stain (Fig. 3B). This difference in β-galactosidase reporter activity is mirrored in an RNA protection assay to detect GAL1 mRNA (Fig. 3C) and Northern blots to detect GAL2,
GAL6 deletion does not affect the stability of GAL1 mRNA. A, an RNA protection assay to measure the degradation of GAL1 mRNA in a GAL6 and a Δgal6 strain after the GAL1 genes were turned off by addition of glucose. Lane 1, GAL1 RNA probe. Lane 2, actin RNA probe. Lanes 3–7, RNA sample from GAL6 and Δgal6 cells 0, 10 20, 30, 40 min after adding glucose to galactose grown cells. Lane 13, probe alone. B, quantitation of A.

As shown above, the negative effect of GAL6 on the GAL gene expression is at the mRNA level. This effect could be through alterations in mRNA production or stability. To distinguish between these two possibilities, we tested the stability of the GAL1 mRNA in a GAL6 wild type and a gal6 deletion strain. Yeast cells were grown in galactose medium to mid-log phase. Glucose was then added to stop the transcription of the galactose-regulated genes. Whole cell RNA was prepared at different times after glucose was added. The RNA samples were subjected to an RNA protection assay to measure the amount of GAL1 mRNA. As shown in Fig. 4, the starting GAL1 mRNA level in a Δgal6 strain is higher than that of a wild type strain as expected from the results reported above, and the rate of decrease of GAL1 mRNA is essentially the same after adding glucose. This indicates that deleting GAL6 has no obvious effect on the stability of GAL1 mRNA at least in this experimental regime. By elimination, it appears that the effect of the GAL6 deletion on GAL1 mRNA levels is on the rate of production rather than the turnover rate of mRNA.

Either of the two known activities of Gal6p, peptidase or nucleic acid-binding, could be responsible for the repression phenotype. To investigate the basis of GAL6-mediated repression, we first created variant forms of the protein by site-directed mutagenesis which had lost either DNA binding or peptidase activity. The peptidase mutant, gal6C73A, was made by replacing the active site Cys73 with alanine. This variant does not have measurable peptidase activity, as assayed against the substrate, N-arginine-7-amido-4-methylcoumarin (Fig. 5B). Disruption of the DNA binding activity of Gal6p protein was accomplished by changing Cys73 to alanine (C73A) has no effect on DNA binding activity, but changing Lys46 and Lys48 to alaines (gal6db) disrupts the DNA binding activity of Gal6p. Radiolabeled single-stranded oligonucleotide UASG1 (1 ng) was used with 20 μg of yeast protein for each assay. Bottom, a Western blot shows that neither mutation has an effect on the level of the protein in the cell. A, a protease assay shows that C73A has no protease activity, but the DNA binding mutant protein (gal6db) maintains its protease activity toward a synthetic substrate Arg7-amido-4-methylcoumarin. C, the protease activity of Gal6p is not required for the negative regulation of the GAL regulon. The gal6db mutant (Δgal6), the wild type (GAL6), and the protease-deficient strain (C73A) were grown in glucose medium at 30°C and then transferred to galactose medium and grown for 30 h. Then cells were harvested and subjected to a β-galactosidase assay. D, the DNA binding activity of Gal6p is not required for the negative regulation of the GAL regulon. Cells were assayed the same way as in C except they were grown for 36 h before being harvested. This may account for the higher activities than in C. The DNA-binding defective Gal6p is designated gal6db.
expression (data not shown). This implies that wild type levels of Gal6p are sufficient to convey full negative regulation. This may not be too surprising considering that Gal6 protein is an abundant protein. We estimate from quantitative Western blots using purified protein as a control that there are ~18,000 Gal6p molecules per cell under glucose growth conditions and 68,000 molecules under inducing (galactose) conditions.

**DISCUSSION**

The yeast form of bleomycin hydrolase has been discovered in several different, apparently unrelated purifications and selections. We now find that it is a galactose-regulated gene. Furthermore, deletion of this gene, **GAL6**, leads to higher expression of **GAL**-regulated genes. This implies that **GAL6** is a newly defined negative regulator of the **GAL** system and part of an autoregulatory circuit.

There is no obvious link between bleomycin hydrolase activity and galactose metabolism. However, **GAL6** is not the first example of a non-galactose metabolism gene under **GAL** control. The **GCY1** gene is also regulated by **GAL4**. It is thought to encode a carbonyl reductase by sequence similarity to animal genes (25). Unlike **GAL6**, however, deletion of **GCY1** has no effect on the cells' ability to grow on galactose or on **GAL** gene regulation. We propose that **GAL6** and **GCY1** may be part of the “environmental” galactose regulon. That is their gene products are not directly involved in the metabolism of galactose, but the presence of galactose generally correlates with some environmental condition that calls for the “environmental” galactose regulon. That is their gene product has a cellular function other than hydroylizing bleomycin.

**Acknowledgments**—We thank Leemor Joshua-Tor, Gary Coombs, Helen Whelan, Clive Slaughter, and the Johnston lab for comments and helpful discussions.

**REFERENCES**

1. Kane, S. A., and Hecht, S. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 113–115
2. Umezawa, H., Maeda, K., Takeuchi, T., and Okami, Y. (1966) *J. Antibiot. (Tokyo)* 19, 200–209
3. Lazo, J. S., and Sebiti, S. M. (1993) *Cancer Chemother. Biol. Response Modif. Annua.* 14, 37–44
4. Ferrando, A. A., Velasco, G., Campo, E., and López-Otín, C. (1996) *Cancer Res.* 56, 1746–1750
5. Akiyama, S., Ikekaki, K., Kuramochi, H., Takashashi, K., and Kuwano, M. (1981) *Biochem. Biophys. Res. Commun.* 101, 55–60
6. Lazo, J. S., and Humphreys, C. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 3064–3068
7. Enenkel, C., and Wolf, D. H. (1993) *J. Biol. Chem.* 268, 7036–7043
8. Magdolen, U., Muller, G., Magdolen, V., and Bandlow, W. (1993) *Biochim. Biophys. Acta* 1171, 299–303
9. Kambouris, N. G., Burke, D. J., and Creutz, C. E. (1992) *J. Biol. Chem.* 267, 21570–21576
10. Xu, H. E., and Johnston, S. A. (1994) *J. Biol. Chem.* 269, 21177–21183
11. Melcher, K. (1997) in *Yeasts* Metabolism (Zimmermann, F. K., ed.) pp. 235–269, Technomeric Publishing Co., Inc., Lancaster, PA
12. Lohr, D., Venkov, P., and Zlatanova, J. (1995) *FASEB J.* 9, 777–787
13. Johnston, M., and Carlson, M. (1992) in *The Molecular Biology of the Yeast Saccharomyces cerevisiae* (Brock, J. R., and Jones, E. W., ed.) Vol. 2, pp. 281–291, Cold Spring Hanbor Laboratory, Cold Spring Harbor, NY
14. Vieira, J., and Messing, J. (1987) *Methods Enzymol.* 153, 3–11
15. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* 2, 165–107
16. Vernet, T., Dignard, D., and Thomas, D. Y. (1987) *Gene* (Amst.) 52, 225–233
17. Myers, A. M., Tzagoloff, A., Kinney, D. M., and Lusty, C. J. (1986) *Gene* (Amst.) 45, 299–310
18. Melcher, K., and Johnston, S. A. (1995) *Mol. Cell. Biol.* 15, 2839–2848
19. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) *Nucleic Acids Res.* 18, 3901–3992
20. Cigan, A. M., and Donahue, T. F. (1987) *Gene* (Amst.) 56, 1–18
21. Brunme, D., Rossi, A. B., Smeekens, S. P., Anderson, D. C., and Payan, D. G. (1996) *Biochemistry* 35, 6706–6714
22. Joshua-Tor, L., Xu, H. E., Johnston, S. A., and Rees, D. C. (1995) *Science* 269, 945–950
23. Pei, Z., and Sebiti, S. M. (1996) *Biochemistry* 35, 10751–10756
24. Leuther, K. R., and Johnston, S. A. (1992) *Science* 256, 1335–1335
25. Magdolen, V., Oechsner, U., Trommler, P., and Bandlow, W. (1990) *Gene* (Amst.) 90, 105–114
26. Bhart, P. J., Oh, D., and Hopper, J. E. (1998) *Genetics* 125, 281–291
27. Torchia, T. E., and Hopper, J. E. (1986) *Genetics* 113, 229–246
28. Nogi, Y. (1986) *J. Bacteriol.* 165, 101–106
29. Torchia, T. E., Hamilton, R. W., Cano, C. L., and Hopper, J. E. (1984) *Mol. Cell. Biol.* 4, 1521–1532
30. Shimada, H., and Fukasawa, T. (1985) *Gene* (Amst.) 39, 1–9
31. Nogi, Y., and Fukasawa, T. (1984) *Nucleic Acids Res.* 12, 9287–9298
32. Igarashi, M., Segawa, T., Nogi, Y., Suzuki, Y., and Fukasawa, T. (1987) *Mol. Gen. Genet.* 207, 273–279
33. Bajwa, W., Torchia, T. E., and Hopper, J. E. (1988) *Mol. Cell. Biol.* 8, 3439–3447
34. Shimada, H., and Fukasawa, T. (1985) *Gene* (Amst.) 39, 1–9