Yeast Dihydroxybutanone Phosphate Synthase, an Enzyme of the Riboflavin Biosynthetic Pathway, Has a Second Unrelated Function in Expression of Mitochondrial Respiration*

Can Jin, Antoni Barrientos, and Alexander Tzagoloff†

From the Department of Biological Sciences, Columbia University, New York, New York 10027

Received for publication, January 19, 2003, and in revised form, February 14, 2003
Published, JBC Papers in Press, February 20, 2003, DOI 10.1074/jbc.M300593200

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

aE280/U1 is a pet mutant of Saccharomyces cerevisiae partially deficient in cytochromes a, a3, and cytochrome b. The ability of this mutant to respire is restored by RIB3, a gene previously shown to code for 3,4-dihydroxy-2-butanone-4-phosphate synthase (DHBP) synthase, an enzyme of the riboflavin biosynthetic pathway. The sequences of RIB3 from wild type and aE280/U1 indicated a single base change resulting in an A137T substitution. The alanine 137 is a conserved residue located in a cavity on the surface of the protein distant from the active site and from the subunit interaction domain involved in homodimer formation. The respiratory defect elicited by this mutation cannot be explained by a flavin insufficiency based on the following evidence: 1) growth of the aE280/U1 on respiratory substrates is not rescued by exogenous riboflavin; 2) the levels of flavin nucleotides are not significantly different in the mutant and wild type. We proposed that in addition to its known function in riboflavin synthesis, RIB3 also functions in expression of mitochondrial respiration. Restoration by riboflavin of growth of a rib3 deletion mutant on glucose but not glycerol/ethanol also supported this conclusion. An antibody against the N-terminal half of DHBP synthase, a gene previously shown to code for 3,4-dihydroxy-2-butanone-4-phosphate synthase (DHBP synthase), catalyzes the first step in the riboflavin biosynthetic pathway (2). Complementation of aE280/U1 by RIB3 was unexpected in view of previous studies indicating that rib3 mutants require riboflavin for growth (3). The lack of correspondence in the phenotype of the rib3 point mutant and a mutant with a complete deletion of RIB3 prompted us to examine the biochemical properties of the mutant further. We present evidence that the phenotype of aE280/U1 is not related to a flavin nucleotide deficiency and propose that in addition to its involvement in riboflavin synthesis, DHBP synthase has another function essential for expression of respiration in yeast.

MATERIALS AND METHODS

Strains and Media—The strains of yeast used in this study are listed in Table I. The respiratory-deficient mutant E280 (MATα met6 rib3) was derived from S. cerevisiae D273-10B/A21 by mutagenesis with nitrosoguanidine (1). The media used for the growth of yeast have been described elsewhere (4). Escherichia coli RR1 was used for amplification of plasmid DNA and for expression of fusion proteins.

Preparation of an Antibody against DHBP Synthase—The region between the BclI and Styl sites coding for the amino-terminal half of DHBP synthase was ligated in frame to the E. coli expression vector pATH2 (7) digested with BamHI. This ligation required the introduction of a BamHI linker at the Styl site. E. coli RR1 cells harboring the plasmid were used to produce the trpE/RIB3 fusion product as described elsewhere (6). Following cells lysis, the overexpressed protein was recovered in the insoluble protein fraction and was dissolved in buffer containing 10 mM Tris-Cl, pH 7.5, 2% SDS, 10 mM β-mercaptoethanol, and 5 μg/ml phenylmethylsulfonyl fluoride. The solubilized fusion protein was further purified by sizing on a Bio-Gel A 0.5 (BioRad) with 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, and 5 mM β-mercaptoethanol as the elution buffer. Fractions enriched for the fusion protein were pooled, concentrated by precipitation with acetone, and dissolved in 0.1% SDS, 1 mM β-mercaptoethanol for immunization of rabbits.

Cloning of the Wild Type and Mutant RIB3—A recombinant plasmid (pG164/T3) containing RIB3 was obtained by transformation of aE280/U1 with a yeast genomic library by the method of Schiestl and Gietz (8). This library, consisting of partial Sau3A fragments of yeast nuclear DNA cloned in the URA3-bearing shuttle plasmid YEp24 (9), was generously provided by Dr. Marion Carlson, Department of Human
DHBP Is Required for Expression of Respiration in Yeast

Table I

| Strain          | Genotype and sources of yeast strains | Source |
|-----------------|--------------------------------------|--------|
| D273-10B/A1    | α met6                               | Ref. 16|
| W303-1A        | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 | R. Rothstein* |
| W303-1B        | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 | R. Rothstein* |
| W303           | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 | W305-1A × W303-1B |
| E280           | ura3-1 rib3                           | Ref. 1 |
| aE280          | ura3-1 rib3;pG164/ST22                | E280 × W303-1A |
| aE280          | ura3-1 rib3;pG164/ST22                | This study |
| aE280          | ura3-1 rib3;pG164/ST22                | This study |
| aW303ARIB3     | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 Δrib3::HIS3 | This study |
| aW303ARIB3     | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 Δrib3::HIS3 | This study |
| aW303ARIB3     | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 Δrib3::HIS3 | This study |
| aW303ARIB3     | ade2-1 his3-1, 15 leu2-3, 112 trp1-1 ura3-1 Δrib3::HIS3 | This study |

* Department of Human Genetics, Columbia University.

Genetics, Columbia University. The chemical method of Maxam and Gilbert (10) was used to sequence the ends of the nuclear DNA insert in pG164/T3.

To clone the mutant rib3 gene, nuclear DNA prepared from aE280/U1 was digested with a combination of PstI and SacI. Fragments averaging 1.9 kb were cloned in YEp352 (11) and used to transform E. coli. The resultant library was screened by colony hybridization with a 32P-labeled 1.9-kb PstI-SacI fragment containing RIB3 (12). The identical procedure was used to clone RIB3 from the wild type strains W303-1A and D273-10B/A1. The two wild type and the mutant RIB3 genes were sequenced by the Sanger method (13) with a commercial sequencing kit (United States Biochemical, Cleveland, OH).

RESULTS

Phenotype of E280 and Identification of the Mutant Gene—

E280 is a respiratory-deficient strain of S. cerevisiae previously assigned to complementation group G164 of a pet mutants collection (1). Like other pet mutants, E280 grows on glucose but not on nonfermentable substrates such as glycerol/ethanol. Some growth of the mutant, however, is observed on glycerol/ethanol after incubation of plates for prolonged periods. The mutant is complemented by pet testers indicating that the expected property of the null mutant is its failure to grow on a medium containing potassium deoxycholate and 1M KCl (19). The difference spectra of the extracts oxidized with potassium ferricyanide and those reduced, respectively, to 25 and 16% of wild type values, indicate a partial deficit of cytochrome oxidase in the mutant was measured (18). Mitochondria were prepared and separated on a 7.5% sucrose gradient (17). Protein concentrations were determined by the method of Lowry et al. (18).

Fig. 1. Phenotype of E280. A, in vivo labeling of mitochondrial translation products. The wild type strain D273-10B/A1 and the rib3 mutant E280 were labeled in the presence of cycloheximide (18). Mitochondria were prepared and separated on a 7.5–15% polyacrylamide gel (20). The ribosomal protein Var1, COX subunits 1 (Cox1), 2 (Cox2), and 3 (Cox3), cytochrome b (Cyt. b), and ATPase subunits 6 (Atp6), and 9 (Atp9) are identified in the margin. B, spectra of mitochondrial cytochromes in the wild type and mutant yeast. Mitochondria were prepared from the respiratory competent parental strain D273-10B/A1 and from the respiratory defective mutant E280. The mitochondria were extracted at a protein concentration of 6 mg/ml in the presence of 1% sodium deoxycholate and 1 M KCl (19). The difference spectra of the extracts oxidized with potassium ferricyanide versus those reduced with sodium dithionite were recorded at room temperature.

To identify the gene responsible for the respiratory defect of E280, the uracil-requiring derivative strain aE280/U1 was transformed with a yeast genomic plasmid library. Two independent respiratory competent clones were obtained from the transformation. The plasmids had overlapping nuclear DNA inserts, one of which (pG164/T3) was used to localize the gene (Fig. 2). The results of subcloning indicated that the gene conferring respiration on aE280/U1 is located between a SacI and a PstI site (Fig. 2). This region of the insert contains RIB3, a gene of chromosome IV, previously shown to code for DHBP synthase (3). This enzyme catalyzes the conversion of ribose-5-phosphate to 3,4-dihydroxy-2-butane-4-phosphate, an early precursor in riboflavin synthesis (2). The failure of pG164/ST31 and pG164/ST32, each containing different halves of RIB3, to restore respiration confirmed the correct identification of the gene. Site directed integration of a single copy of RIB3 in the chromosomal DNA of aE280/U1 restored normal growth on nonfermentable carbon sources (Fig. 3A), suggesting that RIB3 acts by complementation rather than suppression the mutant. aE280/U1 Is a rib3 Mutant—Mutations in RIB3 have been shown to elicit a riboflavin auxotrophy on glucose (3). This phenotype was confirmed in the present study by deleting the gene in the diploid strain W303 (see “Materials and Methods” for the construction of the Δrib3::HIS3 null allele). Growth of the haploid strain W303△RIB3 containing the deletion allele on glucose is strictly dependent on the addition of riboflavin. This phenotype is different from that of E280 or aE280/U1, both of which grow on glucose in the absence of riboflavin. An unexpected property of the null mutant is its failure to grow on a...
Mitochondria were isolated from the parental wild type strain D273-10B/A21 and from the rib3 mutant E280 grown in YPGal and in YPGal supplemented with 25 μg/ml riboflavin. The NADH and succinate oxidase activities were measured at 24 °C as described previously (21). Cytochrome oxidase was also assayed polarographically in the presence of N, N', N'-tetramethyl-p-phenylenediamine and ascorbic acid (21). The values reported are averages of two assays with the ranges indicated.

| Strain          | NADH oxidase (μmol O2/min/mg of protein) | Succinate oxidase (μmol O2/min/mg of protein) | Cytochrome oxidase (μmol O2/min/mg of protein) |
|-----------------|----------------------------------------|---------------------------------------------|---------------------------------------------|
| D273-10B/A21    | 0.50 ± 0.01                            | 0.30 ± 0.00                                 | 0.36 ± 0.01                                 |
| E280            | 0.14 ± 0.02                            | 0.06 ± 0.01                                 | 0.19 ± 0.01                                 |
| E280 + riboflavin| 0.16 ± 0.00                            | 0.05 ± 0.00                                 | 0.13 ± 0.00                                 |

Fig. 2. Restriction map of pG164/T3 and subclones. The restriction sites for BglII (G), PstI (P), SacI (S), XbaI (X), and StyI (St) are indicated on the nuclear DNA insert of pG164/T3. The solid arrow shows the location and direction of the RIB3 open reading frame. The different regions of the insert subcloned in YEp352 are denoted by the bars in the upper part of the figure. The plus and minus signs in parentheses indicate complementation and lack thereof, respectively, of aE280/U1.

nonfermentable carbon source supplemented with riboflavin (Fig. 3A). Like the rib3 null mutant, growth of the point mutants on glycerol/ethanol also fails to be rescued by riboflavin (Fig. 3A).

The discrepancy in the growth properties of the rib3 null mutant and aE280/U1 on glucose fails to be rescued by riboflavin (Fig. 3A).

The ability of a single copy of RIB3 to restore the respiratory deficiency of aE280/U1 and the tight genetic linkage between the mutant allele of aE280/U1 and the rib3 null allele constitute strong evidence that the respiratory deficient phenotype of E280/U1 stems from a point mutation in RIB3.

Sequence of the Mutant RIB3 Gene—To sequence the mutation, the rib3 gene was cloned from aE280/U1. Because this strain was derived from a cross of two non-isogenic strains (E280 and W303-1A), the gene was also isolated from D273-10B/A21 (parent of E280) and W303-1A. The sequences of the rib3 gene of E280 inserted at URA3 and the point mutant aE280/U1 (rib3), the rib3 point mutant aE280/U1/ST22U with RIB3 inserted at URA3 (rib3') and the rib3 point mutant aE280/U1/ST22U with the mutant rib3 gene of E280 inserted at URA3 (rib3''-rib3'), and the null mutant aW303ΔRIB3 with the mutant rib3 gene of E280 (Δrib3''-rib3) or of the wild type gene (Δrib3''-RIB3). The different strains were spotted on rich glucose (YPD) or rich glycerol/ethanol (YPEG) plates with and without 25 μg/ml riboflavin (+ribo). The plates were incubated at 30 °C for 36 h.

2 J. J. Garcia-Ramirez, M. A. Santos, and J. L. Revuelta, GenBank™ accession number Z21619.
from the aE280/U1 mutant, however, had a single G to A transition at nucleotide 409 of the gene, resulting in the substitution of alanine 137 by threonine.

The mutant gene isolated from aE280/U1 was cloned into the integrative plasmid YIp352. Uracl-independent clones obtained by transformation of the rib3 null mutant a/a-W303ΔRIB3 with the E280 gene on the integrative plasmid were sporulated and the growth phenotype of the meiotic products analyzed. The presence of the mutant gene in a single copy restored normal growth of the null strain on glucose and glycerol in the absence of added riboflavin (Fig. 3B). The ability of the mutant gene to rescue the growth of the rib3 null strain is consistent with the growth properties of E280 and suggested that the A137T mutation does not affect riboflavin synthesis. Surprisingly, growth of the point mutant on glycerol was also partially restored when a single copy of the mutant gene was integrated into chromosomal DNA of the null mutant (Fig. 3C).

Flavin Content of Subcellular Fractions from Wild Type and Mutant Cells—The flavin nucleotides and riboflavin compositions of mitochondria and cytosolic fractions (post-mitochondrial supernatant) were compared in the wild type, the mutant aE280/U1, and the mutant transformed with RIB3 on a high copy plasmid. Flavin nucleotides were extracted and separated by HPLC on a C18 column. Although some differences were noted in the amount of riboflavin and flavin nucleotides in the three strains, there was no indication of any significant deficit of FMN or FAD in either the mitochondrial or post-mitochondrial fractions of the mutant (Fig. 4). Some reduction in riboflavin concentration is seen in the post-mitochondrial supernatant fraction of wild type yeast (Fig. 5A). The identity of this protein as DHBP synthase is confirmed by its absence in the rib3 null mutant and its presence at much higher concentrations in the mutant transformed with the wild type gene on a multicopy plasmid. The mass of DHBP synthase derived from the sequence of RIB3 is 22.5 kDa. The reason for the apparent larger mass obtained by SDS-PAGE is not clear.

Although most of the DHBP synthase was detected in the post-mitochondrial supernatant fraction corresponding to the soluble cytosolic proteins of yeast, a small fraction was also present in mitochondria. This was true both for the transformant overexpressing the protein (Fig. 5A) and for wild type (see longer exposure in Fig. 5B). Several lines of evidence indicated that this was not an artifactual association. The mitochondrial fraction isolated from an over-expresser was further purified on a Nycodenz gradient (23). The gradient fractions were probed with antibody against DHBP synthase and cytochrome b2 used here as a mitochondrial marker. The antibody detected two peaks, one of which co-sedimented with the mitochondrial cytochrome b2 marker (Fig. 5C). The second peak was well separated in the upper part of the gradient. No attempts were made to identify the nature of the material in this fraction.

Mitochondria purified on a Nycodenz gradient were diluted under isotonic and hypotonic conditions. Even though the hypotonic treatment causes the outer membrane to burst, it remains attached to and fractionates with the mitoplasts. Both mitochondria and mitoplasts were treated with proteinase K (24). The results of this experiment indicate that disruption of the outer membrane released most of the DHBP synthase and of the intermembrane marker cytochrome b2 (Fig. 5D). The small fraction of the two proteins remaining in the mitoplasts was completely susceptible to the protease. In contrast, DHBP synthase and cytochrome b2 were largely resistant to the protease in unlysed mitochondria (Fig. 5D). The loss of DHBP synthase from the mitoplasts suggests that the protein is located in the intermembrane space. The possibility that DHBP synthase may be associated with the outer membrane and is dissociated from it under hypotonic conditions, however, cannot be excluded.
cytoplasmic concentrations of DHBP synthase are higher in transformants harboring an integrated copy of the gene than in wild type (Fig. 6). This suggests that the chromosomal context (URA3 site in the case of the integrated genes) influences the expression of the gene. The estimated 4–5-fold increase of DHBP synthase in the strain with the integrated mutant allele (aW303ΔRIB3Δ-E280) may explain the ability of this transformant to grow on nonfermentable substrates (Fig. 3B).

**DISCUSSION**

In the present study, the RIB3 gene encoding DHBP synthase, an enzyme of the riboflavin biosynthetic pathway, was cloned based on its ability to restore respiration in the pet mutant, aE280/U1. The rescue by RIB3 is the result of genetic complementation. This is supported by tight linkage of the mutation in aE280/U1 to a rib3 null allele and sequence analysis of the rib3 gene in aE280/U1, which confirmed the presence of a mutation in the gene. The single base mutation causes a relatively innocuous substitution of a threonine for an alanine at residue 137 of the protein.

The rib3 null mutant used in this study shows an auxotrophic requirement of riboflavin for growth on glucose as reported previously (3). In contrast, the rib3 point mutant, like other pet mutants, grows on glucose in the absence of added riboflavin. Moreover, riboflavin does not restore the growth of the mutant on nonfermentable substrates. This suggests that the phenotype of the rib3 point mutant is unrelated to the activity of DHBP synthase in riboflavin synthesis. Instead it must have another function required for expression of mitochondrial respiration. This is also supported by the following evidence. 1) Analysis of FAD and FMN failed to show any substantive decrease in the mitochondrial and cytosolic concentrations of these nucleotides in the mutant aE280/U1. Some decrease in the riboflavin concentration was seen in the cytosolic fraction of the mutant. This, however, could be because of the different genetic background of the two strains used for the analysis. 2) The rates of oxidation of NADH or succinate in isolated mitochondria were not increased when the point mutant was grown in the presence of riboflavin in the growth medium. 3) Riboflavin rescued growth of the rib3 null mutant on glucose but not on nonfermentable substrates.

Transformation of the rib3 null mutant with the rib3 allele of E280 either in single (integrated into chromosomal DNA) or in multiple copies (episomal plasmid) conferred growth on glucose. Surprisingly, the transformants also grew on the nonfermentable substrates glycerol/ethanol. We attribute this to the
elevated level of the mutant protein in the integrant as a result of increased expression of the gene in the new chromosomal context (URA3 locus). This explanation is consistent with the partial restoration of growth of aE280/U1 on glycerol/ethanol when transformed with an extra copy of the mutant gene. These observations together with the slow growth phenotype of aE280/U1 on glycerol/ethanol suggest that the E280 allele reduces but does not abolish the activity of DHBP synthase needed for respiration. Overexpression of the mutant protein, therefore, is able to compensate for the partial loss of function.

The role of DHBP synthase in expression of mitochondrial respiration is not clear. The presence, even at somewhat elevated levels, of riboflavin in the mitochondria of the point mutant excludes DHBP synthase from involvement in riboflavin transport. The presence of riboflavin in the mutant also excludes 3,4-dihydroxy-2-butanone 4-phosphate, the precursor of riboflavin formed by the synthase, from being needed in some other pathway essential for respiration. The more likely alternative is that mitochondrial respiration depends on a function of riboflavin unrelated to riboflavin synthesis. The small fraction of DHBP synthase detected in the mitochondrial intermembrane space suggests that this function is likely to occur in mitochondria.

On the basis of the reported structure of the Magnaporthe grisea DHBP synthase (25), the A137T mutation in aE280/U1 occurs in a region distant from the binding sites for the substrate and metal cofactor. The mutation is also removed from the interface of the homodimer (25). The alanine is located in a depression on the surface of the protein with the methyl group pointing outward. The threonine, with an extra carbon and hydroxyl, can be accommodated without interfering sterically with the neighboring residues in this surface structure. Although the alanine is conserved in the M. grisea enzyme, in some organisms the corresponding position is occupied by serine (26). Conceivably, this domain may be involved in the binding of a molecule that is delivered to mitochondria by DHBP synthase and is required for biogenesis/maintenance of the respiratory chain.

REFERENCES
1. Tzagoloff, A., and Dieckmann, C. L. (1990) Microbiol. Rev. 54, 211–222, 1990
2. Bacher, A., Eberhardt, S., Fischer, M., Kis, K., and Richter, G. (2000) Ann. Rev. Nutr. 20, 153–167
3. Garcia-Remirez, J. J., Santos, M. A., and Revuelta, J. (1995) J. Biol. Chem. 270, 20801–20807
4. Myers, A. M., Pape, L. K., and Tzagoloff, A. (1985) EMBO J. 4, 2097–2092
5. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103–119
6. Rothstein, R. J. (1983) Methods Enzymol., 101, 202–211
7. Koerner, T. J., Hill, J. E., Myers, A. M., and Tzagoloff, A. (1991) Methods Enzymol. 194, 477–490
8. Schiestl, R. H., and Gietz, R. D. (1988) Curr. Genet., 16, 339–346
9. Botstein, D., and Davis, R. W. (1982) in The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 607–636, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 560–564
11. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) Yeast 2, 163–167
12. Feinberg, A. P., and Vogelstein, B. (1985) Anal. Biochem. 132, 6–13
13. Sanger, F., Niblen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Faye, G., Kujawa, C., and Fukuhara, H. (1974) J. Mol. Biol. 88, 185–203
16. Tzagoloff, A., Akai, A., and Needleman, R. (1975) J. Biol. Chem. 250, 8228–8235
17. Wu, M., Repetto, B., Glrum, D. M., and Tzagoloff, A. (1995) Mol. Cell Biol. 15, 264–271
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19. Tzagoloff, A., and Meagher, P. (1972) J. Biol. Chem. 247, 594–603
20. Chua, N. H., and Bennoun, P. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2173–2179
21. Barrientos, A., Korr, D., and Tzagoloff, A. (2002) EMBO J. 21, 43–52
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. Glick, B. S., and Pon, L. A. (1995) Methods Enzymol., 260, 213–223
24. Glick, B. S. (1995) Methods Enzymol. 260, 224–231
25. Liao, D.-I., Zheng, Y.-J., Vitanen, P. V., and Jordan, D. B. (2002) Biochemistry 41, 17951806
26. Stephens, R. S., Kalman, S., Lammel, C. J., Fan, J., Marathe, R., Aravind, L., Mitchell, W. P., Olinger, L., Tatusov, R. L., Zhao, Q., Koonin, E. V., and Davis, R. W. (1998) Science 282, 754–759
Yeast Dihydroxybutanone Phosphate Synthase, an Enzyme of the Riboflavin Biosynthetic Pathway, Has a Second Unrelated Function in Expression of Mitochondrial Respiration

Can Jin, Antoni Barrientos and Alexander Tzagoloff

J. Biol. Chem. 2003, 278:14698-14703.
doi: 10.1074/jbc.M300593200 originally published online February 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300593200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 24 references, 10 of which can be accessed free at http://www.jbc.org/content/278/17/14698.full.html#ref-list-1