

Isolation of the Catalase T Structural Gene of *Saccharomyces cerevisiae* by Functional Complementation

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The catalase T structural gene of *Saccharomyces cerevisiae* was cloned by functional complementation of a mutation causing specific lack of the enzyme (*ctt1*). Catalase T-deficient mutants were obtained by UV mutagenesis of an *S. cerevisiae* strain bearing the *cas1* mutation, which causes insensitivity of catalase T to glucose repression. Since the second catalase protein of *S. cerevisiae*, catalase A, is completely repressed on 10% glucose, catalase T-deficient mutant colonies could be detected under such conditions. A *ctt1* mutant was transformed with an *S. cerevisiae* gene library in plasmid YEp13. Among the catalase T-positive clones, four contained overlapping DNA fragments according to restriction analysis. Hybridization selection of yeast mRNA binding specifically to one of the cloned DNAs, translation of this mRNA in cell-free protein synthesis systems, and demonstration of catalase T protein formation by specific immunoadsorption showed that the catalase T structural gene had been cloned. By subcloning, the gene was located within a 3.5-kilobase *S. cerevisiae* DNA fragment. As in wild-type cells, catalase T synthesis in *ctt1* mutant cells transformed with plasmids containing this fragment is sensitive to glucose repression. By DNA-RNA hybridization, catalase T transcripts were shown to be present in oxygen-adapting cells but absent from heme-deficient cells.

The yeast *Saccharomyces cerevisiae* contains two main catalase proteins called catalase T and catalase A (30, 31). The regulation of these proteins by glucose, oxygen, and heme, their prosthetic group, has been studied by translation in vitro of total yeast mRNA and immunological detection of catalase proteins synthesized by the cell-free system (17, 26). Recently it has been demonstrated that a third hemoprotein of *S. cerevisiae*, iso-1-cytochrome *c*, is also controlled by the three regulators of catalase gene expression (17). Whereas control of iso-1-cytochrome *c* at the transcriptional level has been studied in some detail already (14, 18, 37), no similar information is yet available concerning catalase regulation.

Isolation of the genes coding for yeast catalase is necessary to investigate which of the regulatory effects observed occur at the level of transcription and which DNA regions are involved in control by glucose, oxygen, and heme and to compare these findings with those obtained for iso-1-cytochrome *c*. Cloning of yeast genes has been greatly facilitated by the capability of detecting these genes by complementation of recessive yeast mutations by transformation (4, 15). However, mutations in the structural genes

of yeast catalases have not yet been characterized.

This report describes the cloning of yeast DNA fragments complementing a newly characterized mutation, *ctt1*, which causes specific lack of catalase T. From the results obtained it can be concluded that these DNA fragments contain the catalase T structural gene together with at least some of its regulatory regions.

MATERIALS AND METHODS

***Escherichia coli* and yeast strains; media.** *E. coli* K-12 strain HB101 (*proA2 leuB6 thi-1 lacY1 hsdS20 recA13 rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*) was used in all *E. coli* cloning experiments. The haploid *S. cerevisiae* strains used are listed in Table 1. Yeast strains were routinely grown on YPD medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.], 2% glucose). For analysis of catalase T-deficient mutants, YPD-10% (10% glucose) and YPE (2% ethanol) were used. Yeast transformants were tested for catalase T activity after growth on SD-10% (0.67% yeast nitrogen base [Difco] without amino acids, 10% glucose) supplemented with the appropriate growth requirements. Synthetic media containing 2% ethanol, 0.2% glucose (SE), or 2% raffinose (SR) were used when glucose repression of the cloned catalase T gene was tested.

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
DAS3-1D	α <i>leu1 cas1</i>	(5)
T84	α <i>leu1 cas1 ctt1-1</i>	This study
WS13-9A	α <i>leu2-3 leu2-112 his3 ura3 ctt1-1</i>	This study
DBY747	α <i>leu2-3 leu2-112 his3 trp1 ura3</i>	J. D. Beggs
DczH1-1B	α <i>leu1 ole3</i>	(26)
Dcz8-7c	α <i>leu1</i>	(5)
D273-10B	α	ATCC 24657

Mutagenesis, mutant characterization, and construction of yeast strains. Standard media and procedures were used for mutagenesis, crossing, sporulation, and tetrad analysis (13).

Plasmids and transformation procedures. A DNA library in the *E. coli*-yeast hybrid plasmid YEp13 (8), prepared by partial *Sau3A* digestion of *S. cerevisiae* DNA and cloning into the *Bam*HI site of the vector (22), was obtained from B. D. Hall, University of Washington, Seattle. Plasmids pBR322 (7) and pBR328 (33) were used in subcloning of fragments for hybridization. *E. coli* K-12 strain HB101 was transformed by a slight modification of the procedure described by Cohen et al. (10). *S. cerevisiae* WS13-9A was transformed essentially as described by Beggs (4). Plasmid DNAs were isolated from bacterial cells either over CsCl gradients as described by Clewell (9) or by the rapid alkaline extraction procedure of Birnboim and Doly (6). Plasmid DNA was reisolated from yeast transformants as described by Nasmyth and Reed (21).

Restriction endonuclease digestions. Restriction enzymes and T4 DNA ligase were purchased from BRL, Neu-Isenberg, Germany. Digestions with restriction enzymes were carried out as recommended by the supplier. Restriction fragments were separated electrophoretically in 40 mM Tris-hydrochloride (pH 7.5)–30 mM sodium acetate–1 mM EDTA–0.5 μ g of ethidium bromide per ml with 0.7% agarose gels. Restriction fragments of lambda DNA were used as molecular weight standards. Restriction fragments were reisolated for subcloning as described by Dretzen et al. (12).

Catalase assays. Catalase activities of transformants were detected by a colony test described previously (28). The preparation of cellular extracts and the differential assay for the two yeast catalases using specific antisera were carried out as described previously (35). Protein was determined by the method of Lowry et al. (19).

Hybridization selection of mRNA; in vitro translation. Catalase T mRNA was selected from total yeast RNA isolated as described previously (26) by hybridization to plasmid DNA bound to nitrocellulose filters as described by Ricciardi et al. (25). Hybridized RNA was released by boiling and was translated in the presence of L-[³⁵S]methionine (The Radiochemical Centre, Amersham, United Kingdom; 600 Ci/mmol) in mRNA-dependent cell-free protein synthesis systems from wheat germ (27) or *S. cerevisiae* (16). Characterization of translation products by immunoadsorption with catalase antibodies, dodecyl sulfate-polyacryl-

amide gel electrophoresis, and detection of labeled proteins by fluorography were done as described previously (1, 2).

DNA-DNA and DNA-RNA hybridizations. Total yeast DNA was isolated as described by Olson et al. (23), digested with restriction enzymes, and transferred from agarose gels to nitrocellulose as described by Southern (34). Polyadenylated [poly(A)⁺] RNA for hybridization experiments was prepared from total RNA by oligodeoxythymidylic acid-cellulose chromatography (3). Denaturation of RNA with glyoxal-dimethyl sulfoxide, agarose gel electrophoresis of denatured RNA, transfer to nitrocellulose, and hybridization were carried out as described by Thomas (36). Plasmid DNAs used as hybridization probes were ³²P labeled by nick translation as described by Maniatis et al. (20) by using a nick translation kit obtained from The Radiochemical Centre.

RESULTS

Mutant isolation and characterization. To obtain catalase T-deficient mutants, *S. cerevisiae* DAS3-1D was grown to the stationary phase on YPD-10%. Cells were plated on solid YPD-10% (10⁴ cells per plate). Plates were UV irradiated (2,000 ergs/mm²; 1% survival) and were then incubated at 30°C. Crude extracts obtained from glucose-grown cells show no catalase A activity as long as some glucose is still present in the medium (11, 29). It was expected, therefore, that no catalase A activity would be detectable in colonies (28) grown on plates containing 10% glucose. The results described below show that this assumption was justified. Further, strain DAS3-1D bears the *cas1* mutation, which causes insensitivity of catalase T, but not of catalase A, to glucose repression (32). Therefore, well-grown master plates were replica plated on YPD-10%. After growth overnight, colonies were covered with 3% H₂O₂. Colonies not forming O₂ bubbles were isolated from the master plate and retested for catalase activity after growth on YPD-10% and YPE. Absence of catalase T after growth on liquid YPE was tested in crude extracts by polyacrylamide gel electrophoresis followed by specific staining for catalase activity (35).

This procedure led to the isolation of three mutants (T84, T104, T136) lacking catalase T activity and belonging to the same complementation group. The mutation responsible for the lack of catalase T activity was designated *ctt1*. The allele in mutant T84, which was used in further studies, was designated *ctt1-1*. In crosses with wild-type strains, the *ctt1* mutation was recessive and exhibited 2:2 segregation during tetrad analysis. No evidence for centromere linkage or linkage to genes already mapped was obtained. Further attempts to map the *ctt1* mutation are currently in progress.

Transformation and selection of catalase T-

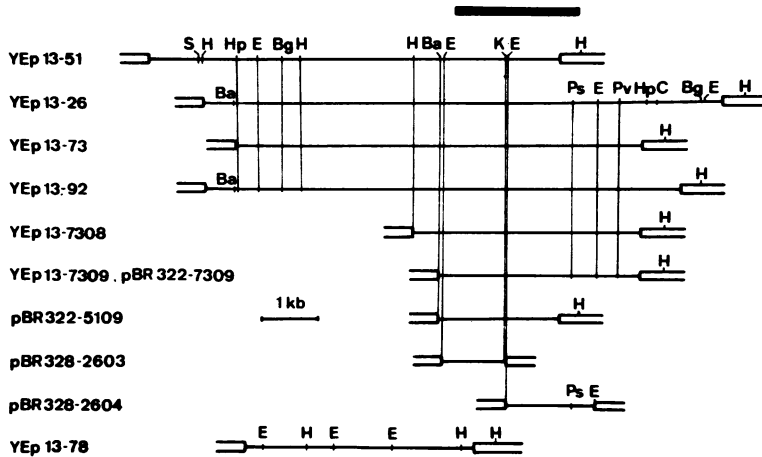


FIG. 1. Restriction maps of inserts of cloned plasmids. E, *EcoRI*; H, *HindIII*; Ps, *PstI*; Ba, *BamHI*; S, *SphI*; Bg, *BglII*; Pv, *PvuI*; C, *ClaI*; Hp, *HpaI*; K, *KpnI*. No restriction sites were detected for *XhoI*, *Sall*, or *AvaI*. Insert sequences are indicated by lines; vector sequences are indicated by open bars. The approximate position of the catalase T gene is indicated by a closed bar. Only *EcoRI* and *HindIII* sites were mapped in the case of plasmid YEp13-78. Size bar represents 1 kilobase (kb).

positive transformants. *S. cerevisiae* T84 was crossed with strain DBY747 to obtain segregants bearing both *ctt1* and *leu2* mutations. Appropriate segregants were transformed with vector DNA to test which segregant could be transformed most efficiently. Strain WS13-9A, which was transformed as efficiently as strain DBY747, was used for all further studies. To clone the catalase T gene, strain WS13-9A was transformed with 3 μ g of the yeast DNA pool (22) in vector YEp13 containing the yeast *LEU2* gene as selectable marker. Approximately 10^4 transformants were selected on plates lacking leucine. Transformant colonies were transferred to SD-10% plates supplemented with histidine and uracil. Colonies were grown at 28°C and were replica plated to the same type of plates. Colonies on one set of plates were then covered with 3% H_2O_2 and were visually tested for catalase activity. Seven catalase-positive transformants were isolated. Retesting after streaking clones for single colonies, preparation of cell-free extracts, and differential testing for catalase activities with specific antisera demonstrated that three transformant clones produced catalase T (see also Table 2). As shown with the help of specific antisera, a fourth transformant clone, YEp13-78, gave rise to production of catalase A after growth on media containing 10% glucose (data not shown). A fifth clone, YEp13-51, was catalase positive when tested on plates, but its extracts were inactive.

Characterization of *S. cerevisiae* transformants. All transformants obtained were mitotically unstable. Under nonselective conditions (growth on YPD medium), cells lost the ability to synthesize catalase T at a rate of approxi-

mately 3% per generation. Loss of catalase T was always accompanied by simultaneous loss of the ability to grow in the absence of leucine.

For further characterization, plasmid DNAs were isolated from the catalase-positive yeast transformants. *E. coli* cells were transformed with the DNA preparations and selected for ampicillin-resistant clones. Plasmids amplified in this manner were reisolated and tested for the ability to complement the *leu2* and *ctt1* mutations of *S. cerevisiae* WS13-9A. Plasmids derived from the four original yeast transformants producing catalase T and plasmid YEp13-78 complemented both mutations. These plasmid DNAs were characterized further by restriction analysis. The results are summarized in Fig. 1. Four of the five plasmids contain overlapping insertions (YEp13-51, YEp13-26, YEp13-73, YEp13-92). In the overlapping parts of the four insertions, identical restriction patterns were observed in all cases, except for the left end of the insertion of YEp13-51, which may be a cloning artifact. According to restriction analysis (Fig. 1), plasmid YEp13-78 is unrelated to the other four clones obtained. Further, preliminary characterization of the cloned yeast catalase A gene (G. Cohen and H. Ruis, unpublished experiments) indicates that the insert of plasmid YEp13-78 does not contain this gene.

Identification of the cloned gene by mRNA hybridization selection. To obtain direct proof of the presence of the catalase T structural gene on the cloned DNAs, one of them was tested for its ability to bind catalase T mRNA specifically under conditions allowing DNA-RNA hybridization. Following the procedure described by Ricciardi et al. (25), plasmid YEp13-73 was linear-

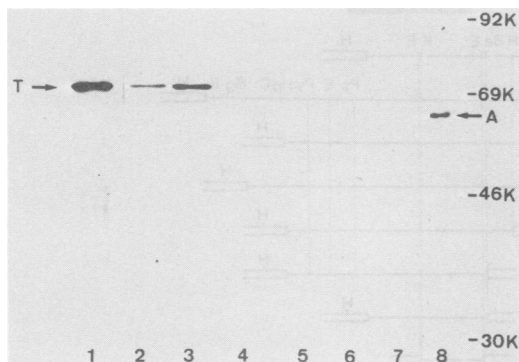


FIG. 2. Hybridization selection and in vitro translation of catalase T mRNA. Total yeast RNA was hybridized to linearized plasmids bound to nitrocellulose. Experiments involved translation in a wheat germ system [lanes 1, 2, and 4 through 8] or in a cell-free yeast system (lane 3). Dodecyl sulfate-polyacrylamide gel electrophoresis after immunoabsorption was carried out with anti-catalase T (lanes 1 through 6) or with anti-catalase A (lanes 7 and 8). Gels show translation of: lanes 1 and 8, total yeast mRNA from O_2 -adapting strain D273-10B; lanes 2, 3, and 7, mRNA from O_2 -adapting cells hybridized to YEp13-73; lane 4, mRNA from strain DczH1-1B grown in the absence of δ -aminolevulinic acid (26) hybridized to YEp13-73; lane 5, mRNA from O_2 -adapting cells hybridized to pBR322; lane 6, mRNA from O_2 -adapting cells hybridized to YEp13. Molecular weight markers used were phosphorylase *b* (92,000), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). T and A show positions of catalase T and catalase A, respectively, labeled in vivo.

ized by treatment with *Xho*I and was bound to nitrocellulose filters. Control experiments were carried out with the vector, YEp13, linearized with *Xho*I, and with plasmid pBR322, which was linearized with *Hind*III. Total yeast mRNA isolated from cells adapting to oxygen for 45 min was used for hybridization to the filter-bound DNAs since a relatively high amount of catalase mRNAs is found in such cells (17). Control hybridizations were carried out with mRNA isolated from the heme-deficient mutant DczH1-1B. This mutant has previously been shown to virtually lack translatable catalase T mRNA (17, 26). mRNA bound to nitrocellulose filters was freed by boiling and was translated in a cell-free protein synthesis system from wheat germ. In addition, mRNA from oxygen-adapting cells hybridizing with plasmid YEp13-73 was translated in an mRNA-dependent translation system from yeast cells. Catalase proteins synthesized were isolated by immunoabsorption with specific antisera directed against catalase T or catalase A and were characterized further by dodecyl sulfate-polyacrylamide gel electrophoresis. The results are summarized in Fig. 2. They show that catalase T mRNA was bound to filters loaded

with plasmid YEp13-73 but not to filters containing control DNAs. No protein band migrating like catalase T was detected with RNA from heme-deficient cells, and no binding of catalase A mRNA to plasmid YEp13-73 was observed.

Tentative localization of catalase T gene on cloned DNAs. Cells of *S. cerevisiae* WS13-9A transformed with the four original plasmids bearing the catalase T gene and with plasmids obtained by subcloning (YEp13-7308 and YEp13-7309; Fig. 1) were grown on different media under conditions of glucose repression and under derepressing conditions. Soluble extracts obtained from cells were tested for catalase T activity. The results are summarized in Table 2. Extracts of cells transformed with all plasmids except for YEp13-51 showed catalase T activity. Even in the case of the smallest subclone, YEp13-7309, catalase T formation was sensitive to glucose repression. The glucose repression effect observed was quantitatively similar to that observed in wild-type strains (11, 29). It can be concluded, therefore, that the 3.5-kilobase insert of this plasmid contains the catalase T structural gene and at least some of its regulatory sequences.

Extracts from cells transformed with plasmid YEp13-51 lacked catalase T activity completely, whereas the transformants clearly showed catalase activity in a colony test. From this finding, it was tentatively concluded that this plasmid contains an incomplete catalase T gene coding for an altered catalase T protein that is sufficiently stable in intact cells but unstable in extracts. The approximate position of the catalase T gene derived from this observation, from the subcloning data, from the size of the protein (subunit molecular weight of in vitro translation product, 69,000) and from the size of the transcript of the gene (see Fig. 4) are indicated in Fig. 1.

Southern analysis. Hybridization of yeast ge-

TABLE 2. Catalase T activities of transformants

Plasmid	Activity of extract obtained from cells grown on ^a :		
	10% Glucose	2% Raffinose	2% Ethanol
YEp13-26	3.2	12.3	17.9
YEp13-92	4.3	12.0	36.3
YEp13-51	0	ND ^b	0
YEp13-73	2.3	11.1	22.7
YEp13-7308	2.3	ND	19.6
YEp13-7309	2.9	9.9	28.4
YEp13	0	ND	0

^a Activity is expressed as micromoles of H_2O_2 decomposed per minute per milligram of protein (11). Cells were grown on synthetic medium to an optical density at 600 nm of 2.0.

^b ND, Not determined.

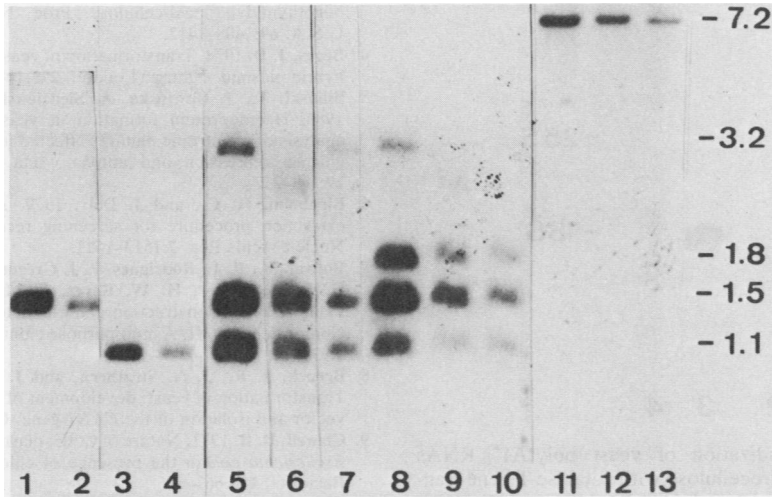


FIG. 3. Southern blot autoradiogram of total yeast DNAs hybridized with catalase T gene fragments. DNA from strain D273-10B (lanes 1, 3, 6, 9, and 12), Dcz8-7C (lanes 2, 4, 5, 8, and 11), or WS13-9A (lanes 7, 10, and 13) was digested with *EcoRI* (lanes 1 through 10) or *BglIII* (lanes 11 through 13) and hybridized after electrophoresis and blotting to ^{32}P -labeled plasmid pBR328-2604 (lanes 1 and 2), pBR328-2603 (lanes 3 and 4), pBR322-5109 (lanes 5 through 7 and 11 through 13), or pBR322-7309 (lanes 8 through 10). Fragment lengths are indicated in kilobases.

nomic DNA isolated from two wild-type strains, *S. cerevisiae* Dcz8-7C and D273-10B, and from the *ctl1* mutant WS13-9A was carried out with probes derived from the cloned fragments. The results (Fig. 3) are in line with the restriction maps of the cloned DNAs except for one *EcoRI* fragment, which, in the DNA from strain D273-10B, is different. This observation is probably explained by a restriction site polymorphism, which is located outside of the DNA region relevant to studies of the catalase T gene. Not surprisingly, hybridization of the 3.2-kilobase *EcoRI* fragments in Fig. 3, lanes 5 to 8 and 10, and of the corresponding smaller fragments in lanes 6 and 9 is quite weak because of the minimal overlap of the labeled probes with these restriction fragments.

Analysis of transcripts. Poly(A)⁺ RNA from oxygen-adapting cells and from the heme-deficient mutant DczH1-1B were transferred to nitrocellulose after agarose gel electrophoresis and were hybridized with two labeled probes containing DNA from the catalase T gene region. In the case of RNA from oxygen-adapting cells, a single band migrating like 18S rRNA was observed with both probes (Fig. 4). This band was undetectable with RNA from heme-deficient cells.

DISCUSSION

The results of this paper show that the catalase T gene was isolated by complementation of the *ctl1* mutation. This conclusion is mainly based on the results of mRNA hybridization selection followed by in vitro translation. It

could be shown that one of the cloned DNAs specifically selects an mRNA from total yeast RNA, which, upon in vitro translation, gives rise to a product identified as catalase T protein by immunoadsorption with specific antibodies and by electrophoresis. This specific mRNA is not bound to the cloned DNA from an RNA preparation isolated from heme-deficient cells. Such RNA preparations have previously been shown by direct in vitro translation to lack translatable catalase T mRNA. No indication of cross-hybridization of the catalase T gene with sequences coding for catalase A, the second catalase protein of *S. cerevisiae*, was obtained in DNA-RNA hybridization (mRNA hybrid selection or hybridization of probes containing parts of the catalase T gene with RNA blotted to nitrocellulose). This result is in agreement with the conclusion drawn earlier (1) that there is no precursor-product relationship between catalase T and catalase A. The result also provides evidence for the existence of two different structural genes coding for the two proteins.

Up to now, only limited results have been obtained with the help of the cloned gene concerning the regulation of catalase T. Analysis of transformants shows that these have retained glucose repression sensitivity of catalase T despite the fact that they contain the gene in a multicopy plasmid. Previous results have shown that levels of translatable catalase T mRNA are controlled by glucose, oxygen, and heme (17, 26). The DNA-RNA hybridization data presented in this paper indicate that at least some of these differences in levels of translatable cata-

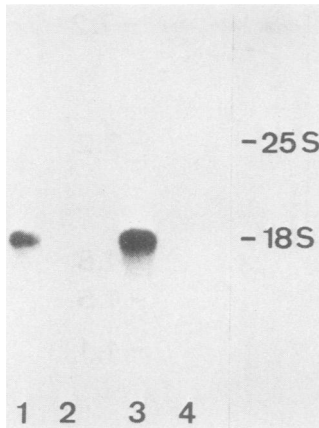


FIG. 4. Hybridization of yeast poly(A)⁺ RNAs transferred to nitrocellulose with catalase T gene fragments. Lanes 1 and 3, Poly(A)⁺ RNA from strain D273-10B grown anaerobically and adapted to O₂ for 45 min; lanes 2 and 4, poly(A)⁺ RNA from strain DczH1-1B grown in the absence of δ-aminolevulinic (26). Hybridization was performed with labeled plasmid pBR328-2603 (lanes 1 and 2) or pBR328-2604 (lanes 3 and 4). Positions of cytoplasmic yeast ribosomal RNAs are given as size markers.

lase T mRNA are equivalent to differences in amounts of catalase T mRNA sequences present in the cells. More extensive and more systematic studies are necessary, however, to substantiate this conclusion.

The nature of the *ctt1* mutation characterized and used in this investigation has not yet been clarified with absolute certainty. Its specificity and lack of apparent leakiness has led to the working hypothesis that it is a catalase T structural gene mutation. Successful isolation of this gene by complementation of the mutation adds another strong argument in favor of this hypothesis. Final proof for its correctness will have to be obtained by integration (24) of the cloned catalase T gene at the chromosomal *ctt1* site.

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