The Yeast *his4* Multifunctional Protein

IMMUNOCHEMISTRY OF THE WILD TYPE PROTEIN AND ALTERED FORMS*

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A procedure for rapid purification of the yeast *his4* protein has been developed. A combined biochemical and immunological study of the *his4* proteins from wild type and a number of mutant strains indicates that the *his4* gene product is a trifunctional protein with three independent functional domains. Active fragments of the *his4* protein possessing one or several activities can result from proteolysis or from genetic alteration. Such fragments can be purified to homogeneity by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Immunological experiments with antibody to the wild type protein and with antibody to genetically or proteolytically altered forms indicate that the sole product of the wild type *his4* region is a 95,000-dalton protein. Furthermore, such data indicate that each mutant protein has a characteristic stability that correlates with the type of mutation and with the location of the mutation within the *his4* locus. By varying the ionic strength of the extraction buffer, it is possible to find conditions under which such mutant proteins are quite stable.

Rapid purification of the *his4* gene product allowed us to demonstrate that all three activities specified by this region are associated with a single multifunctional polypeptide of 95,000 daltons. In previous studies, examination of partially purified preparations and various alternative purification procedures had given indications of other molecular forms of the protein. In addition, nonsense mutations at various positions within the *his4C* region were previously found to produce *his4* proteins of reduced molecular weight (2). In this report we show that each of the alternative forms of the protein is missing one or more of the three *his4* activities.

We have used immunological procedures to elucidate the relationship between these altered forms of the protein and the 95K1 protein. The results confirm earlier work showing that the 95K protein is the initial gene product of the *his4* region (3, 4). Furthermore, the alternative forms of the protein produced in crude extracts or during lengthy purification procedures are not present in *vivo*; they are created by proteolysis in *vivo*. The *his4* protein in *vivo* is a single multifunctional polypeptide. Since active fragments can be formed as a result of mutation or proteolysis, we conclude that the trifunctional protein possesses discrete, independent functional domains. Procedures for stabilizing the mutant proteins together with the use of antibodies elicited to these mutant *his4* protein species permitted the analysis of the structures of mutationally altered forms of the protein.

**EXPERIMENTAL PROCEDURES**

*Reagents—* Amino acids, dithiothreitol, EDTA, Tris-HCl, sodium azide, Triton X-100, guanidine-HCl, agarose, phenazine methosulfate, Coomassie brilliant blue R, nitro blue tetrazolium, NAD, and 100-μM beads were obtained from Sigma. Acrylamide, bis-acrylamide, N,N',N',N'-tetramethylethylenediamine, and pyrazole were products of Eastman. Glycerol, methanol, MnCl₂, MgCl₂, and boric acid were obtained from Mallinckrodt Chemical Works. Schwarz/Mann (NH₄)₂SO₄, and urea were used. Sepharose 4B and protein A were products of Pharmacia. CNBr and bromphenol blue were from Aldrich. N-AM-P-Sepharose was obtained from P-L Biochemical Corp., and DE52 and phosphocellulose were from Whatman. Glycerine was obtained from Nutritional Biochemical Corp., SDS was from Pierce, and Nonidet P-40 detergent was from Shell Chemicals. The reagent, 2-p-iodo-3-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride, used in the histidinol dehydrogenase assay was purchased from Dajac Laboratories. Histidinol was a product of Bachem Fine Chemicals. Braun was the manufacturer of the 500-μm glass beads (54170) used in cell disruption. Ammonium persulfate was a product of Bio-Rad Laboratories. Radioiodinated protein A was obtained from Amersham. Bovine serum albumin was a product of Pentex, while other molecular weight standards were from Pharmacia. All microbiological media, along with Freund's complete and incomplete adjuvant, were obtained from Difco. [35S]Methionine was a gift of R. F. Gesteland of the Cold Spring Harbor Laboratory.

*Strains—* The derivation of the *S. cerevisiae* strains used in this study has been described elsewhere (5-8). Strain B7 was obtained from D. H. Wolf, Biochemisches Institut der Universität, Freiburg im Breisgau, W. Germany. *Staphylococcus aureus* Cowan I (ATCC 12598) bearing protein A was provided by W. C. Summers, Department of Radiology, Yale University.

*Growth and Preparation of Cells—* Cells were grown at 30 °C in yeast nitrogen base synthetic medium (9) supplemented with 0.20 mM histidine, when necessary, either batchwise in a New Brunswick gyratory shaker-incubator in 2-liter flasks or in a 25-liter New Brunswick fermentor. When a cell density of about 5 × 10⁷ cells/ml was attained, cells were harvested, washed, diluted 2.5-fold, and then grown in minimal medium for an additional 6 h to elevate the histidine biosynthetic enzymes. Harvested yeast cells were washed and used immediately or stored overnight on ice before disruption. Large scale extracts were obtained as described below.

To survey the cross-reactivity of *his4* protein in extracts of mutant strains under various conditions, cells were grown as above. A modification of a multisample cell breakage device (10) constructed by B. DeTroy at Cold Spring Harbor Laboratory was employed using an amount of buffer equal to the harvested cell weight, 3-fold volume of 500-μM glass beads, and a treatment time of 4 min. Extracts were

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clarified by centrifugation in adapters for 10 min at 18,000 × g and subjected to immunodiffusion in agarose or immunoprecipitation with IgG.

Assays—Histidinol dehydrogenase his4C activity was measured by a colorimetric assay (11, 12). The absorbance at 520 nm was read in a Zeiss PMII spectrophotometer. Protein concentration was determined by the method of Lowry et al. (13). Crystalline bovine serum albumin was served as a standard.

Electrophoresis—Gels of 10% polyacrylamide containing 0.1% SDS were prepared according to the method of Laemmli (14). Gels were fixed in 50% methanol, 7% acetic acid containing 0.2% Coomassie blue and destained in 5% methanol, 7.5% acetic acid. Electrophoresis sample buffer contained 0.08 M Tris-HCl (pH 6.8), 0.1 M dithiothreitol, 2.0% SDS, 10% glycerol, and 0.2% bromphenol blue. Gels were vacuum-dried on a hot plate prior to autoradiography. Analytical gels were run in 10% polyacrylamide gels containing 0.1% agarose in 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl. Approximately 15 μl of solution filled a well.

Antibody Purification—In order to obtain the immunoglobulin fraction of the immune rabbit serum, thawed serum was combined with an equal amount of neutralized saturated (NH₄)₂SO₄ solution, stirred for 30 min at 0−5 °C, and centrifuged for 10 min at 18,000 × g. The pellet was then redissolved in 0.025 M potassium phosphate (pH 7.2) 1% glycerol. The solution was brought to 40% saturation by the addition of two-thirds of volume of a saturated (NH₄)₂SO₄ solution, stirred for 30 min, and centrifuged as before. The pellet was dissolved in a buffer volume of approximately one-tenth the original volume of serum and was rapidly dialyzed against 0.025 M potassium phosphate (pH 7.2), 1% glycerol with 2 changes. After less than 4 h of dialysis, the buffer volume was replaced with the original volume and stored overnight at room temperature.

Purification of the his4 Protein—Yeast grown in a 25-liter fermentor yielded 5–6 g of cells/liter. Yeast cells harvested by centrifugation were resuspended in an equal amount of 0.05 M Tris-HCl (pH 7.5) and disrupted in a Braun homogenizer for 3 min in 30-s bursts using an amount of glass beads equal to 3 times the volume of resuspended yeast cells. All procedures were carried out at 0−5 °C unless otherwise specified. Cell debris was removed by centrifugation for 10 min at 27,000 × g. The supernatant was brought to 50% saturation by the addition of solid (NH₄)₂SO₄, stirred for 30 min, and then centrifuged as before. The 50−70% fraction was resuspended in 0.05 M Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.01 mM L-histidinol, 10 mM MgCl₂, 1.0 mM MnCl₂, 5% glycerol (Buffer A) and dialyzed against this buffer overnight. The dialysis sample of 10 ml was poured into 20 ml of electrophoresis sample buffer. Buffer A was employed as described elsewhere (4) to purify the (NH₄)₂SO₄ fraction further. Routinely the N'-AMP-Sepharose was recrystallized by washing with 0.02 M Tris-HCl (pH 7.5), 4.0 M guanidine-HCl and then Buffer A. After washing with Buffer A and Buffer containing 0.10 M NaCl, bound protein including the 3 his4 activities was eluted with Buffer C containing 1.0 M NaCl. Fractions containing maximal histidinol dehydrogenase activity were pooled, dialyzed against 0.05 M Tris-HCl (pH 7.5), 0.10 M NaCl, 0.01 mM histidinol, 1 mM dithiothreitol, 5% glycerol (Buffer B) overnight, and then applied to a DEAE-cellulose column (1.7 × 15 cm). The column was washed extensively with Buffer B containing 0.10 M NaCl. His4 activities were released with Buffer B containing 0.3 M NaCl. Peak fractions were dialyzed against 0.01 M (NH₄)HCO₃ and then lyophilized. The lyophilized preparation was dissolved in electrophoresis sample buffer, boiled for 2 min, and then applied to a large preparative 10% polyacrylamide-SDS slab gel. The major species, the 95,000-dalton his4 protein, was sliced out of the gel and stored at −20 °C. The gel slices were washed with electrophoresis buffer in test tubes and placed in 10-ml disposable pipettes plugged at the bottom. Dialysis bags were slipped onto the tips of the pipettes. The protein was eluted from the gel by electrophoresis for 24–48 h at 250–300 V at 0−5 °C. Eluted protein was lyophilized, dialyzed against 0.01 M (NH₄)HCO₃ and then precipitated with 80% acetone containing 1 mM HCl. The protein was again boiled in 0.01 M (NH₄)HCO₃. This procedure was repeated to electrophoresis once more and the 95,000-dalton protein was excised from the gel and eluted electrophoretically. After dialysis against PBS, this homogeneous protein preparation was used to immunize rabbits.

Immunization Procedure. Female New Zealand White rabbits were immunized subcutaneously with a homogenous preparation of his4 protein. A 0.5-ml preparation containing 2.0 mg of his4 protein was dialed against PBS and emulsified with a syringe with an equal volume of Freund’s complete adjuvant. The emulsion was centrifuged to remove bubbles and 0.2-ml aliquots were injected subcutaneously in rabbits. After 3 week, 3 rabbits were boosted by subcutaneous injection with the same amount of material but in Freund’s incomplete adjuvant. After 10–14 days immune serum was collected via the marginal ear vein. Approximately 2–3 weeks after the second injection, rabbits were boosted intravenously with 200 μg of his4 protein in 1.0 ml of PBS. Preimmune serum had been collected and stored at −20 °C prior to immunization.

Collected blood was allowed to stand for 30 min at room temperature and then stored at 0–5 °C overnight. Serum was separated from the clot by centrifugation in a clinical centrifuge for 10 min at 2,000 × g. The supernatant was stored frozen at −20 °C. Serum was tested by double-diffusion analysis overnight at room temperature. Ouchterlony (16) agarose slabs contained 1% agarose in 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl. Approximately 15 μl of solution filled a well.

Preimmune serum was then combined with 30 μg/ml of his4 protein, dialyzed against Buffer C, clarified by centrifugation, and stored frozen at −20 °C. The protein concentration of the IgG preparation was 30 mg/ml.

Immunoprecipitation Procedures—The standard immunoprecipitation procedure employed 0.10 ml of yeast crude extract and 20 μl of purified IgG preparation. Both preparations were buffered with 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 1% glycerol (Buffer C). After incubation overnight on ice the mixture was centrifuged for 10 min at 2,000 × g and the supernatant was discarded. The precipitate was washed 3 times with 0.20 ml of 0.10 M Tris-HCl (pH 7.5), 0.20 M NaCl, 2% Triton X-100, 2.0% urea, 2% glycerol, and then once with Buffer C. Supernatant was removed gently without perturbing the pellet. During the washing steps the resuspended pellet was vortexed vigorously and then centrifuged. The final precipitate was drained and 0.10 ml of electrophoresis sample buffer was added. The sample was boiled for 2 min and routinely 10 μl was applied to a 10% polyacrylamide, 0.1% SDS gel. An identical procedure could be employed with unfractionated serum, although a smaller amount of antigen was immunoprecipitated. In all cases, preimmune serum did not result in the immunoprecipitation of yeast proteins.

Analysis of Polyethylenimine—Preimmune serum and the 3 his4 protein antigen were performed using staphylococcal absorbant. S. aureus Cowan I cells bearing protein A (17) were grown and prepared as described by Kessler (18). Cells were stored as a 10% (v/v) suspension in 0.04 M potassium phosphate (pH 7.2), 0.15 M NaCl, 0.05% sodium azide at 0–5 °C. Within 24 h of use, the bacterial cells were centrifuged at 2,000 × g for 10 min and incubated in 0.5% Nonidet-P-40 detergent, 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.05 M EDTA, 0.02% sodium azide for 15 min at room temperature. Cells were washed in the above buffer but with 0.05% NP-40 and then resuspended in this buffer.

53S-labeled cells were grown in sidearm Klett flasks using standard procedures. Each 40-ml culture of wild type or mutant strain contained 13 μCi/ml of 35S]methionine. Cell-free extracts contained approximately equal amounts of radiolabel. A volume of 0.10 ml of extract was incubated with 20 μl of IgG of antibody raised to the his4 protein and 25 μg of purified unlabeled his4 protein for 2 h on ice before addition of S. aureus cells. A volume of treated cell suspension equal to one-tenth of the antibody-extract volume was used. After 1 h, the cell-free extracts were boiled for 2 min at 10,000 × g and the cells were washed 4 times with 1.0 ml of the Triton-urea buffer described above. After a final wash with Buffer C the staphylococcal absorbant was boiled for 2 min in 20 μl of electrophoresis sample buffer to release bound 35S-antigen and IgG and stored at −20 °C. The absorbant was carefully removed and then applied to a polyacrylamide-SDS gel. After electrophoresis, the gel was dried and autoradiographed.
Preparation and Use of IgG-Sepharose 4B—The purified immunoglobulin fraction of antibody raised to the his4 protein was coupled to Sepharose 4B by the CNBr activation procedure of Cuatrecasas (19). Approximately 5 mg of IgG was added/ml of bed volume of Sepharose in 0.01 m potassium phosphate (pH 7.3), 0.35% NaCl. After coupling, the IgG-Sepharose was washed copiously with Buffer C.

To investigate the retention of antigen, yeast crude extracts prepared in Buffer C were applied at room temperature to the IgG-Sepharose equilibrated with the same Tris-saline buffer. The column was then washed extensively at 0–5 °C and the bound protein was released at room temperature with Buffer C containing 2% SDS. Columns were washed with Buffer C immediately after elution of bound protein and later re-used. IgG-Sepharose was stored in Buffer C containing 0.1% sodium azide.

**Purification of the 60,000-dalton Proteolytic Fragment**—A purified proteolytic fragment was derived from the native 95,000-dalton protein by treatment with a yeast extract containing high protease activity. The yeast extract was obtained from yeast mutant B7 which has elevated levels of proteases A and B (20). The 95,000-dalton protein for these experiments was purified by (NH4)2SO4
crystallization and then fractionation on DEAE-cellulose and valine-Sepharose as described under “Results.” Protein purified in this way was stored and converted in 0.05 m Tris-HCl (pH 7.5) containing 30% glycerol at −20 °C. A small amount of protein (2%/v) prepared from strain B7 fractionated in the same way was added to this partially purified preparation. Conversion of histidinol dehydrogenase to a second form was monitored by staining native gels for his4C activity. The pattern was compared with the pattern produced by a preparation without added B7 extract. After an incubation period the treated extract produced a band of histidinol dehydrogenase activity that migrated more rapidly on the nondenaturing gel than did the untreated extract. When all of the visible activity had been converted to the altered form, the preparation was subjected to preparative electrophoresis in a native Tris-borate gel. The regions that displayed histidinol dehydrogenase activity in the dye-gel assay were excised and the protein contained within the slice was eluted electrophoretically and repurified in an SDS-polyacrylamide gel. Eluted 60K protein was lyophilized and washed twice with 80% acetone, 1 mm HCl. The final homogeneous 60K protein was injected subcutaneously into rabbits and these injections were followed by intravenous immunization. In both injections 100 μg of antigen was used. Immunological procedures were the same as those described for antibody raised to the 95,000-dalton his4 protein.

Valine-Sepharose 4B was derivatized according to the method of Cuatrecasas (19) and was used as a hydrophobic matrix employing procedures described elsewhere (21).

**RESULTS**

Location of Mutations in the his4 Region.—The his4 gene cluster encodes enzyme activities catalyzing steps 3, 2, and 10 of histidine biosynthesis. Fig. 1 is a map of the his4ABC region describing the relative locations of nonsense, frameshift, missense, and deletion mutations. Earlier work led to the characterization of these mutations and their relative locations within the his4 gene cluster (5–8).

**Purification of the his4 Protein for Immunological Studies**—The purification procedure was simplified in order to obtain large amounts of his4 protein. As discussed under “Experimental Procedures” the simplified purification involved passage of a 50–70% (NH4)2SO4 fraction over AMP-Sepharose, followed by chromatography on DEAE-cellulose. The final steps were preparative electrophoresis in SDS-polyacrylamide gels followed by electrophoretic elution of the his4 protein from the polyacrylamide. One hundred g of yeast yielded approximately 5 mg of purified his4 protein.

**Antibody to the his4 Protein**—In order to obtain antibody to the purified his4 protein, rabbits were immunized and immune rabbit serum was collected. The antibody was tested on Ouchterlony double-diffusion slides using crude serum (Fig. 2). Crude extracts of a wild type strain and of strains with missense mutations A588 or B331 produced distinct precipitin lines. The antibody was highly specific. No cross-reaction was apparent when extracts of strains bearing nonsense mutation A385 or A86 or deletion 15 were examined. Preimmune serum did not react with yeast crude extracts.

Immune serum specifically removed the 3 his4 enzyme activities from the supernatant of a partially purified preparation (4). In addition, active histidinol dehydrogenase-antibody complexes could be detected in immunodiffusion agar employing a coupled dye system that measured histidinol dehydrogenase activity. The specificity of this reaction was demonstrated by its dependence on L-histidinol and by its dependence on antigen with a genetically undamaged his4C region (22).

**Initial Purification Procedure: Proteolytic Conversion of the his4 Protein**—The initial purification procedure employed for the yeast his4 protein, which was modified to obtain large amounts of his4 protein for immunization, involved fractionation with (NH4)2SO4 affinity chromatography, hydrophobic chromatography, and chromatography on DEAE-cellulose. The final steps were activity staining followed by electrophoretic elution of the protein from the stained gels. This procedure purified the 95K his4 protein but promoted proteolytic conversion. An extract was prepared as described under “Experimental Procedures” and the 50–70% (NH4)2SO4 fraction was passed over N+,-AMP-Sepharose. Peak fractions of histidinol dehydrogenase activity were concentrated by (NH4)2SO4 precipitation. The preparation was dialyzed against 0.05 m Tris-HCl (pH 7.5), 1.0 m (NH4)2SO4 and then

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**Fig. 1. Genetic maps of the his4 region.** a, the relative locations of missense mutations; b, the relative locations of frameshift mutations (above the line) and nonsense mutations (below the line); c, the relative locations of deletions. A, B, and C, functional segments.
applied to a valine-Sepharose column (21). The his4 activities were retained under high (NH₄)₂SO₄ conditions and were released by lowering the ionic strength with 0.05 M Tris-HCl (pH 7.5), 30% glycerol. Eluted protein was dialyzed against Buffer B containing 0.1 M NaCl and subjected to chromatography on DEAE-cellulose. The protein fraction released from DEAE-cellulose was concentrated by (NH₄)₂SO₄ fractionation and then stained for his4C activity in a nondenaturing 10% polyacrylamide-Tris-borate gel using a nitro blue tetrazolium/phenazine methosulfate dye system. The reactive regions were excised and the protein was eluted electrophoretically using the gel system of Laemml (14). This purification procedure yielded a single species in an SDS-polyacrylamide gel. Employing protein standards of known molecular weight the his4 protein was calculated to have a Mₛ of 95,000.

Sensitivity of the his4 protein to proteolytic degradation could be demonstrated during staining for his4C activity (Fig. 3). In a fresh preparation (Fig. 3, experiment a) the predominant species was form I, this form migrating more slowly during electrophoresis. Two faint bands appeared below the major staining region after a longer incubation period. After 2 weeks of storage in glycerol at −20 °C a second species, form II, was evident. The intensity of the new band was equal to that of the form I band (Fig. 3, experiment b). In some cases endogenous alcohol dehydrogenase activity in the preparation caused a reaction of the dye system. However, pyrazole, a potent inhibitor of alcohol dehydrogenase activity in such gel assays when present at a concentration of 1 mg/ml, did not affect the pattern in Fig. 3. After storage for 4 weeks, the conversion to form II was complete. Only the lower band of his4C activity was visible (Fig. 3, experiment c). The second form of the his4 protein has been shown to lack his4A and his4B activities and, based on sucrose density gradient centrifugation of a partially purified preparation, it has been estimated to be about 60,000 daltons in molecular mass (23).

The proteolytic fragment appeared in aged crude extracts or after certain column steps, especially after hydrophobic chromatography on valine-Sepharose. Most likely under high ionic strength conditions the valine-Sepharose step removed one or more protease inhibitors, thereby activating the several yeast proteases. Activity staining of a partially purified preparation from B7, a strain which lacks an inhibitor of protease A, revealed a rapid conversion to form II. The conversion (from form I to form II) was enhanced when some of this preparation from B7 was added to wild type protein. Strain B7 has been shown to possess elevated levels of proteinases A and B (20).

The proteolytic fragment was purified as described under “Experimental Procedures” using a partially purified extract of mutant B7 to promote conversion to the second form of the his4 protein. Based on polyacrylamide-SDS gel electrophoresis using standards of known molecular weight the fragment was determined to have a Mₛ of 60,000. Since the direction of transcription is known for the his4ABC cluster (2), this peptide bearing the fragment with his4C activity possesses the C-terminus of the his4 protein.

The purified 95K his4 protein and purified 60K proteolytic fragment displayed immunological identity based on the Ouchterlony double-diffusion pattern in Fig. 4. Furthermore, antibody to the 95K protein immunoprecipitated the purified 60K proteolytic fragment. SDS-gel electrophoresis of the immunoprecipitate revealed a band directly above the 50K band corresponding to the heavy chain of IgG.

**Fig. 4. Ouchterlony immunodiffusion pattern obtained with purified 95K his4 protein and purified 60K proteolytic fragment.** The upper wells contained the purified proteins. The center well contained IgG purified from antibody raised to the his4 protein. The pattern was stained with Coomassie blue.

**Fig. 3. A nondenaturing gel of partially purified his4 protein stained for histidinol dehydrogenase activity.** Experiment a employed a fresh preparation where the predominant species was form I: 1, 10 min incubation; 2, 20 min incubation. Experiment b employed an aged preparation illustrating the conversion to form II: 1, with pyrazole; 2, without pyrazole. Experiment c employed a preparation stored until the conversion to form II was complete. Incubation time was 10 min.
Fig. 5 (left). Ten per cent polyacrylamide gel of proteins removed from yeast crude extracts by IgG of antibody raised to the his4 protein. 1, purified 95K his4 protein; 2, protein removed from a crude extract of a mutant strain with mutation A385 by IgG-Sepharose; 3, protein removed from a wild type crude extract by IgG-Sepharose; 4, protein immunoprecipitated from a crude extract of a strain with mutation A385 by IgG; 5, protein immunoprecipitated from a wild type crude extract by IgG.

Fig. 6 (center). Ten per cent polyacrylamide-SDS gel of purified his4 proteins: the wild type species and two altered forms. The 95K wild type protein is on the left. The 60K species is the proteolytic fragment with his4C activity. The right channel contains the 45K ochre nonsense fragment, a mutant protein from a strain still possessing A and B activities.

Fig. 7 (right). Three his4 protein species immunoprecipitated by IgG of antibody raised to the his4 protein and resolved in a 10% polyacrylamide-SDS gel. Channels on the left contain IgG only and purified 95K his4 protein. Immunoprecipitation experiments were performed with a wild type crude extract, with an aged wild type crude extract (*), and with a partially purified extract from a strain with mutation C864.
contain antigenic material that could be retained on the antibody column (Fig. 5). All nonsense mutants examined were found to lack the 95,000-dalton his4 protein based on retention studies with IgG-Sepharose. Neither IgG nor IgG-Sepharose removed alternative forms of the his4 protein from freshly prepared crude extracts. The 60K proteolytic fragment could not be detected in either experiment.

Purification of Ochre Nonsense Fragment C864—Peptide fragments of the his4 protein generated by nonsense, frameshift, or deletion mutations are exceptionally unstable (22, 24, 25). The enzyme activity and immunological activity of these fragments, bearing one or more of the activities of the his4 region, could not be easily detected. Specific protease inhibitors did not have a marked effect on fragment stability. Cross-reacting material in extracts of strains with nonsense, frameshift, or deletion mutations could be detected neither by conventional immunoprecipitation procedures using in vitro 35S-labeled protein released from staphylococcal absorbant, by in vitro protein synthesis using mRNA from various mutant strains, nor by immunodiffusion analysis using yeast crude extracts in Tris-saline. Radioiodinated IgG from immune serum raised to the his4 protein has proved useful in detecting minute amounts of the labile fragments by immunoprecipitation analysis (25). Glycerol and (NH4)2SO4 are also useful; both agents have a marked effect on fragment stability. This action may be a result of the inhibition of proteases or it may be an effect on the conformational state of the fragment, which reduces its susceptibility to proteolysis (24, 26). Observations with extracts of a strain bearing lesion C864 indicated that high ionic strength in (NH4)2SO4 had a greater stabilizing effect than did glycerol. In order to stabilize his4 protein fragments, the stabilization buffer employed routinely for cell disruption was 0.10 M Tris-HCL (pH 7.5), 1.0 M (NH4)2SO4, 30% glycerol. Such conditions had proved especially useful in the detection and sizing of extremely labile his4 nonsense fragments by immunoautoradiography of polyacrylamide-SDS gels (24).

Of all the mutant strains examined, one with nonsense mutation C864, a UAA mutation that lies in the middle of the his4 region, yielded the most stable termination fragment. High ionic strength conditions were used to stabilize and purify this ochre nonsense fragment. Approximately 75 g of cells were disrupted in stabilization buffer. The crude extract was clarified by centrifugation for 10 min at 27,000 × g. Immediately afterwards the extract was made 80% saturated with respect to (NH4)2SO4 by the addition of a saturated solution. After centrifugation the supernatant was discarded. The pellet was resuspended in 0.05 M Tris-HCl (pH 7.5), 30% saturated in (NH4)2SO4. The precipitated protein obtained after centrifugation was discarded. Saturated (NH4)2SO4 solution was added to 70% of saturation. The preparation was centrifuged and the resulting pellet was resuspended in Buffer C. The 30-70% salt fraction was then dialyzed against 1 liter of Buffer C with one change for 3 h. The dialyzed preparation, 9.0 ml in volume, was then mixed with 11.0 ml of the immunoglobulin fraction purified from immune rabbit serum raised to the his4 protein. After incubation on ice overnight, the preparation was centrifuged for 10 min at 27,000 × g and the immunoprecipitated protein was washed 3 times with 4.0 ml of the Triton/urea buffer described above and twice with 5.0 ml of Buffer C. The final precipitate was boiled in electrophoresis sample buffer. After preparative polyacrylamide-SDS gel electrophoresis, an intense major band was apparent below the 50K band representing the heavy chain of IgG. The band was cut out of the SDS gel and the protein was eluted electrophoretically from the gel slice. Approximately 4 mg of purified C864 nonsense fragment was obtained. Electrophoresis in an SDS-gel calibrated with proteins of known molecular weight indicated that the termination fragment bearing A and B activities has a M, of 45,000. This result was in agreement with the value obtained by sucrose density gradient centrifugation of a crude extract from a strain with mutation C864 whereby the location of the his4A and his4B activities in the gradient was determined relative to Escherichia coli alkaline phosphatase (2).

Antibody was raised to the 45K ochre nonsense fragment using the same methods that were employed to obtain antibody to the his4 protein. The immunoglobulin fraction was purified from immune serum as before.

Fig. 6 shows a 10% polyacrylamide-SDS gel of the 3 his4 proteins that have been purified, the 95K protein and the two altered forms, the 60K proteolytic fragment with his4C activity and the 45K ochre nonsense fragment with his4AB activities. Sequencing studies of the his4 region indicate that the 45K nonsense fragment possesses the NH2-terminus of the his4 protein (27).

Immunoprecipitation of 3 his4 Protein Species with Antibody to the 95K Protein—Antibody to the 95K protein immunoprecipitated the wild type protein as well as mutant and proteolytically altered forms. Immunoprecipitation experiments with crude extracts were performed using IgG purified from immune serum raised to the 95K his4 protein (Fig. 7). Several different extracts were examined in these experiments. SDS-gel electrophoresis of proteins immunoprecipitated from a crude extract of a wild type strain revealed a distinct 95K band. The same experiment but with an aged crude extract resulted in the appearance of a 60K band above the band representing the heavy chain of IgG. An extract of a strain with mutation his4-654 was prepared in a high salt buffer and concentrated by (NH4)2SO4 precipitation. SDS-gel electrophoresis of proteins immunoprecipitated from this preparation revealed a 45K band.

Immunoprecipitations with Antibodies to the 3 Purified his4 Protein Species—Antibody raised to each of the altered forms immunoprecipitated the 95K protein (Fig. 8). The purified IgG fraction of immune serum was used in each case. When crude extracts of a strain with polar mutation A385 were used as the source of antigen, no yeast proteins were precipitated.

IgG of antibody raised to the 60K proteolytic fragment immunoprecipitated the purified 95K his4 protein. In addition, antibody to the 60K protein immunoprecipitated the his4 protein from crude extracts of strains with missense mutations his4A-588 or his4B-331. The antibody failed to recognize the C864 fragment.

IgG of antibody raised to the 45K C864 nonsense fragment also immunoprecipitated missense A and missense B proteins from crude extracts. In addition it recognized the labile C864 fragment. Experiments with extracts of strains with nonsense mutations A385 or A86 did not yield immunoprecipitated proteins by SDS-gel analysis or precipitin lines after immunodiffusion.

The Immunological Response of Missense C or Nonsense C his4 Proteins—Extracts of strains with missense C mutations 280, 207, or 290 failed to cross-react with antibody to the 96K his4 protein based on Ouchterlony analysis. Furthermore, while SDS-gels of immunoprecipitates of extracts of strains with missense A or B mutations revealed a 95K band, experiments with IgG and missense C extracts did not show the band after electrophoresis in SDS-polyacrylamide gels (Fig. 9). Such missense C strains, however, still possessed his4A and his4B activities. The high salt stabilization buffer employed for fragments generated by deletions and nonsense or frameshift mutations was very effective in stabilizing the
immunological activity of these missense C his4 proteins. When this stabilization buffer was used precipitin lines were apparent and an immunoprecipitated 95K protein could be seen in an SDS-gel (Fig. 9). Extracts of missense C strains prepared in 0.05 M Tris-HCl (pH 7.5), 1.0 M (NH₄)₂SO₄, 30% glycerol, contained immunological activity. Missense C proteins appeared either especially sensitive to proteolytic degradation or were unable to attain conformational states exposing antigenic sites that could be recognized by antibody.

In control experiments, extracts of strains with polar nonsense A mutations 385 and 86 or polar frameshift A mutations 38 or 519 prepared in conventional or high salt buffer lacked cross-reacting material based on immunodiffusion, immunoprecipitation, or immunoelectrophoresis. Partially purified 50-70% (NH₄)₂SO₄ fractions of such mutant extracts also lacked antigenic material. Fragments generated by nonsense mutations C52, C864, and C1175 were barely detectable by double-diffusion but could be detected if the extracts were stabilized by high salt conditions (Fig. 10) or after immunoelectrophoresis (24). The fragment generated by frameshift mutation C712 could also be detected by immunodiffusion and immunoelectrophoresis after such extraction procedures. In summary, proteins produced by strains with frameshift, nonsense, and missense mutations in his4C appeared unstable based on several methods of analysis. All 3 classes of mutant proteins generated by mutations in the C region of the his4 locus were more stable in buffer of high ionic strength. Immunoprecipitations with [³⁵S]Methionine-labeled Yeast Crude Extracts—³⁵S-labelling experiments were performed to determine whether the degraded forms of his4

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Fig. 8 (left). Ten percent polyacrylamide-SDS gel of proteins immunoprecipitated from crude extracts of wild type yeast and extracts of a strain with nonsense mutation A385. Three antibody preparations were employed: IgG of antibody raised to the 95K wild type protein monomer, IgG of antibody raised to the 60K proteolytic fragment, and IgG of antibody raised to the ochre nonsense termination fragment.

Fig. 9 (right). Missense hist4 proteins immunoprecipitated with antibody to the hist4 protein. Crude extracts and IgG were employed. Immunoprecipitated proteins were subjected to electrophoresis in a 10% polyacrylamide-SDS gel. a, immunoprecipitation with extracts prepared in Tris-NaCl of strains bearing nonsense mutations B331, A588, A1111, B594 or missense C mutations C207, C280, or C290. Controls were performed with extracts of strains bearing polar nonsense mutations A25 or A385. Channels on the right contained IgG only or hist4 protein only. b, proteins immunoprecipitated from an extract of a strain with missense mutation C280 after extraction in high salt buffer and partial purification.

Fig. 10. Ouchterlony immunodiffusion slide of yeast extracts prepared under high salt conditions and antibody to the hist4 protein. The strains employed were wild type and 2 mutant strains with nonsense C mutations 864 and 32.

Fig. 11. SDS-polyacrylamide gel autoradiogram of immunoprecipitated [³⁵S]methionine-labeled wild type and mutant his4 proteins. Labeled crude extracts from wild type, from nonsense A385, C32, and C864 strains, and from a missense B331 strain were employed. IgG purified from antibody raised to the his4 protein and staphylococcal immunoabsorbent were included in the reaction mixture along with unlabeled purified his4 protein as carrier. The labeled proteins were released from the immunoabsorbant, resolved in a 10% polyacrylamide-SDS gel, and autoradiographed. The exposure time was 12 h.

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R. Bigelis and K. Burridge, unpublished data.
Yeast hist4 Multifunctional Protein

5151

protein were present in the cell. The immunoprecipitation experiments were repeated utilizing [35S]methionine-labeled crude extracts of wild type yeast and mutant strains. IgG purified from antibody raised to the hist4 protein was added to a labeled crude extract along with unlabeled purified hist4 protein and staphylococcal adsorbant bearing protein A on the cell surface. After incubation on ice, immunoprecipitated 35S-labeled proteins were released from the immunoadsorbant and resolved in a 10% polyacrylamide-SDS gel (Fig. 11). Autoradiography revealed an intense band in the channels containing immunoprecipitated proteins from a wild type strain and a strain with missense mutation B321. The bands corresponded exactly with unlabeled hist4 protein that was immunoprecipitated and were 95,000 in molecular weight based on protein standards. No major protein bands were apparent when 35S-labeled nonsense mutant extracts were employed. Crude extracts of mutants with lesions hist4-A385, hist4-C52, or hist4-C864 did not contain a 95,000-dalton protein. The wild type crude extract did not contain the 60K species, the altered protein produced by proteolysis but still recognized by antibody, demonstrating the absence of any endogenous 60K hist4C fragment. These observations with 35S-labeled yeast proteins emphasized the specificity of the antibody raised to the hist4 protein and indicated that the 95K protein was the sole in vivo product of the hist4 region.

The procedure using staphylococcal adsorbant was quite important in detecting labeled hist4 protein. The method allowed the vigorous washing of the adsorbant with relatively large volumes of the Triton/urea buffer prior to release of label. A similar procedure has allowed the detection of [35S]-methionine-labeled hist4 protein after cell-free translation of crude yeast mRNA using mammalian in vitro protein synthesis factors. Crude mRNA from a strain with the polar mutation A385 did not produce a [35S]methionine-labeled 95K protein in the in vitro system.

DISCUSSION

The combined genetic, biochemical, and immunological approach presented in this work indicates that the yeast hist4 region encodes a single, multifunctional protein of 95,000 daltons bearing 3 histidine biosynthetic activities. These observations agree with results obtained with the purified native protein (4), also indicating that it is a trifunctional polypeptide. Immunological data are consistent with the determination of the multifunctional nature of the hist4 protein. Only one protein was apparent in wild type extracts after immunoprecipitation and SDS-gel electrophoresis or immunautoradiography (24). The 95K protein was absent in each nonsense mutant extract investigated. Antibody to a hist4C peptide and antibody to a hist4AB peptide immunoprecipitated the 95K species only in the case of wild type or nonsense strains. No other yeast proteins were immunoprecipitated when a strain with the most proximal nonsense mutation (hist4AB, A385) was used. All altered species were absent from wild type extracts.

In the functional independence of the 3 hist4 loci led to several models for the product of the hist4 region. The detection of 1 or 2 of the remaining activities in mutants with nonsense mutations in the C locus or deletions in the A or B loci demonstrated that components of the hist4 enzyme complex could function independently of the other activities specified by the hist4 region (2, 7). Complementation patterns and genetic studies were consistent with these results. Missense mutations did not abolish more than one activity, although they did affect the levels of activity of the 2 remaining enzymatic functions (5, 6). The information available made it difficult to decide between a 1 gene-3 domains hypothesis as compared with a 3 genes-3 polypeptides hypothesis. This work demonstrates that only one polypeptide is the product of the hist4 gene. Active fragments of the hist4 protein can result from mutations. Based on this biochemical data, it is apparent that the presence of independent domains is responsible for the apparent functional independence.

Active fragments of the hist4 protein can also result from proteolysis. The observation of proteolytic conversion supports the notion of independent functional domains of the hist4 protein. A hist4C proteolytic fragment of the hist4 protein still possessed histidinol dehydrogenase activity, though it lacked about one-third of the hist4 protein. It is conceivable that the protein possesses structural regions that separate the trifunctional protein into discrete functional units and that such linking sequences are especially sensitive to proteolytic cleavage after cell disruption. Mutant B7, a strain having elevated levels of protease A and B (20), displayed an increased rate of conversion in extracts, as did preparations of yeast hist4 protein from wild type containing some of the B7 extract. Our evidence indicates that such proteolytic processes are not physiologically significant. Only the 95K protein and none of the degradation products could be detected in fresh extracts of a wild type strain by immunoprecipitation or by immunautoradiography.

All yeast hist4 protein fragments generated by mutations were unstable. Incomplete polypeptides displayed differential stability that was not correlated with size but was most likely dependent on conformation. These observations appear similar to those made with bacterial systems (28-31). Comparable observations have been made with in vivo β-galactosidase fragments in E. coli (29). Certain mutant hist4 proteins were especially unstable. For example, the incomplete polypeptide caused by mutation C1176 was far more unstable than the incomplete polypeptide caused by mutation C864 or other nonsense mutations, even though mutation C1176 is the most distal mutation available in the hist4 region. Thus, stability did not correlate with fragment size. Mutation C1176 was originally described as a strong antipolar mutation since it resulted in the removal of his4A and his4B enzyme activities (7). All missense C proteins tested were exceptionally unstable, as were certain nonsense C proteins.

The general instability of certain classes of mutant proteins is not surprising considering the level of proteolytic activity in yeast crude extracts (32). However, the uniqueness of hist4 proteins with altered C regions of the polypeptide suggests that improper folding of histidinol dehydrogenase exposes sites susceptible to cleavage and results in rapid proteolytic degradation. High ionic strength dramatically increased the half-life of nonsense fragments and allowed the purification of the fragment generated by mutation C864. High ionic strength conditions have been shown to reduce proteolysis (32). Such ionic strength conditions have also been shown to stabilize and cause conformational changes in α-isopropylmalate isomerase, another yeast amino acid biosynthetic enzyme that catalyzes multiple reactions (26).

The hist4 region is not polycistrionic like the bacterial his operon. No examples of operons have been found in yeast. A possible difference between eukaryotic and prokaryotic translation systems may be the inability of eukaryotic ribosomes to recognize the internal initiation signals of polycistrionic mRNA. Mutations in intercistrionic regions could have resulted in gene fusion and may be the mechanism for the evolution of multifunctional proteins such as the yeast hist4 protein. The hisD and hisC genes of Salmonella typhimurium encoding histidinol dehydrogenase and aminotransferase, have been fused by mutation (33). The product of the fused
Yeast hist4 Multifunctional Protein

gene is a bifunctional protein. The selective advantage in yeast for the fusion of the genes encoding activities catalyzing 3 nonsequential steps is unclear. A large polypeptide could act as an organizing center for the assembly or localization of the remaining enzymes of histidine biosynthesis. Such complexes could more efficiently catalyze the sequence of histidine biosynthetic reactions thereby “channeling” (34, 35) intermediates. Thus, multifunctional proteins may play an important catalytic and organizational role in eukaryotes.

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R Bigelis, J K Keesey and G R Fink

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Regulation of insulin receptor metabolism. Differentiation-induced alteration of receptor synthesis and degradation.

Brent C. Reed, Gabriele V. Ronnett, Peter R. Clements, and M. Daniel Lane

Page 3924, Table I, line 1:
Under \( k_d \), 0.5 should be 0.05 h\(^{-1}\)

On the mechanism of ribonucleoside triphosphate reductase from \textit{Lactobacillus leichmannii}. Evidence for 3'-C-H bond cleavage.

JoAnne Stubbe, Deborah Ackles, Raj Sehgal, and Raymond L. Blakley

The third author's name should be Raj K. Sehgal

The effect of detergent selection on retinal outer segment \( A_{280}/A_{300} \) ratios.

David G. McConnell, Charles A. Dangler, Deborah M. Eadie, and Burton J. Litman

Page 4913, Summary, line 22:
Due to a printer error "1%" was omitted before detergent. The correct sentence should read:

At 11 \( \mu \)M rhodopsin and 1% detergent concentrations, the 3 nonionic detergents displayed negligible \( A_{280} \) and no significant sediment upon centrifugation.

The yeast \textit{his4} multifunctional protein. Immunochromatography of the wild type protein and altered forms.

Ramunas Bigelis, Joseph K. Keesey, and Gerald R. Fink

Page 5150, Fig. 10:

Fig. 10 is reprinted here from a new original print supplied by the author: