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# THE IMMUNOLOGICAL DETECTION OF YEAST NONSENSE TERMINATION FRAGMENTS ON SODIUM DODECYLSULFATE-POLYACRYLAMIDE GELS

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Summary. A sensitive immunological technique is described that detects proteins in sodium dodecylsulfate gels of crude cell extracts. The method is based on the binding of $^{125}$I-protein A to gels that have been incubated with antibody to a specific protein. Using antibody to the yeast HIS4 protein, single polypeptides can be detected in mutant and wild-type extracts. The size of these polypeptides correlates both with the type of mutation and with its location in the HIS4 region.

## Introduction

The HIS4ABC region of Saccharomyces cerevisiae codes for the enzyme activities catalyzing steps 3, 2 and 10 of histidine biosynthesis (1,2). Recently it was shown that the HIS4ABC region encodes a single 95,000 dalton multifunctional protein that bears all three enzyme activities (3). Extensive genetic studies with strains bearing deletion, nonsense, or frameshift mutations in this region indicate that active fragments may occur in such mutants (4,5). However, work with these mutants has been hindered by the instability of the genetically altered proteins, which in most cases has prevented their identification and purification. In this paper we describe the identification of mutant proteins of the HIS4 region using a modified, sensitive immunological technique (6) to detect the proteins in SDS[1]-polyacrylamide gels of crude cell extracts. The size of the polypeptides detected correlates both with the type of mutation and its relative position in the HIS4 cluster. The determination of the size of these fragments confirms the recent finding that the wild-type protein is multifunctional and contains all three activities.

[1] Abbreviations used are: SDS, sodium dodecylsulfate; BSA, bovine serum albumin.

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## Materials and Methods

Liter cultures of cells were grown in yeast nitrogen base synthetic medium (7) at 30°C supplemented with 20 mg histidine to a density of about 250 Klett units (No. 52 filter), harvested, washed, resuspended to a cell density of 100 Klett units, and then grown in minimal medium for 6 hr to elevate the histidine biosynthetic enzymes. Unless otherwise specified procedures were carried out at 0-5°C. Harvested cells were resuspended in an equal volume of 0.05 M Tris-HCl, pH 7.5, 1.0 M $(NH_4)_2SO_4$, 30% glycerol and disrupted in a Braun homogenizer for 2 min using a 3 times volume of 100 micron glass beads. Cell debris was removed by centrifugation for 10 min at 27,000xg. Crude extract was immediately mixed with an equal volume of electrophoresis sample buffer (0.08 M Tris-HCl, pH 6.8, 0.1 M dithiothreitol, 2.0% SDS, 10% glycerol, and 0.2% bromophenol blue), boiled for 2 min, rapidly frozen in a $CO_2$-ethanol bath, and stored at -20°C. Approximately 10 µg of protein from each strain was applied to 10% polyacrylamide-SDS gels prepared according to the method of Laemmli (8). After electrophoresis gels were fixed for 2 hr in 5:5:1 water:methanol:acetic acid, and then washed in excess buffer A (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% $NaN_3$) until the pH returned to pH 7.5. The gels were overlaid with 2 to 3 ml of antibody preparation (dilution 1:40) to the HIS4 protein (R. Bigelis and G.R. Fink, unpublished data) containing 3.0 mg per ml protein to which an additional 5.0 mg per ml BSA was added. The gels were incubated under high humidity at room temperature for 12 hr. Antibody was the immunoglobulin fraction prepared by passage of an $(NH_4)_2SO_4$ fraction of immune rabbit serum over DEAE-cellulose and phosphocellulose columns. After incubation with the first antibody the gels were washed in buffer A for at least 2 days before being overlaid with $^{125}$I-protein A (specific activity approximately 0.5mCi per mg of protein and used at 5µCi per ml). BSA was added at 5.0 mg per ml and the gels were incubated for 12 hr as before. After the second incubation the gels were washed for a further 2 days in buffer A before drying and autoradiography.

Staphylococcal protein A (Pharmacia) was iodinated using the Bolton-Hunter reagent (9,10). The protein A was dialyzed against 0.1 M borate buffer, pH 8.5. One mg of protein A in 0.1 ml of buffer was added to 1 millicurie of the dried Bolton-Hunter reagent (New England Nuclear) and the reaction was allowed to proceed at 0°C for 1 hour with periodic shaking. The unchanged ester was reacted with 0.2 M glycine in 0.1 M borate buffer, pH 8.5, for 5 minutes. The iodinated protein A was then separated from the other labelled reaction products by chromatography on Sephadex G50 (Pharmacia, fine grade). The iodinated protein A had a specific activity of 0.5 millicuries per mg protein. It was stored frozen in aliquots together with carrier protein (BSA) at 5 mg per ml.

## Results

The genetic map shown in Figure 1 specifies the locations of the six mutations used in this study. Both missense mutations A588 and B331 allow the expression of the remaining two HIS4 functions. Strains bearing nonsense mutations C864, C52, or C1176, retain HIS4A and HIS4B enzymatic activities. A strain with the polar nonsense lesion A385, however, no longer possesses any of the three activities produced by the gene cluster (4).

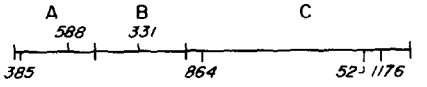


Figure 1. Location of mutations in the HIS4 region. The solid line represents a portion of yeast chromosome III. A, B, and C designate functional segments specifying steps, 3, 2, and 10 of histidine biosynthesis. Mutations above the line are missense mutations, while those below the line are nonsense mutations. The strains bearing these HIS4 lesions have been described by G.R. Fink (1,2,4,5).

An antiserum raised against the recently purified HIS4 95,000 dalton protein was used to study mutant polypeptides of the HIS4 region. Of many nonsense strains examined only the 45,000 dalton C864 fragment could be immunoprecipitated in sufficient quantities to allow visualization in an SDS-gel stained with Coomassie blue. We used, therefore, a modification of the technique for detecting immunologically reactive polypeptides directly in SDS-gels (6) and thereby reduced to a minimum the time available for proteolytic degradation of unstable genetic fragments. Crude cell extracts were dissolved immediately in SDS sample buffer and subjected to electrophoresis in polyacrylamide-SDS gels. After fixation, the resulting gels were reacted first with the antibody directed against the wild-type HIS4 protein and then, after washing, with radio-iodinated protein A, a Staphylococcus aureus cell surface protein that binds to IgG of many species (11). Reactive antigens were detected by autoradiography and typical autoradiograms are shown in Figure 2. A single 95,000 dalton polypeptide is detected in gels of crude extracts from the wild-type and the two missense mutants examined here. These co-migrate with the purified HIS4 protein. When the extract from a mutant with an early nonsense mutation, A385, was examined no reactive polypeptides were found. However, three nonsense mutations in the C region each produced polypeptides with altered molecular weights. From gels calibrated with molecular weight standards we calculate that the nonsense mutations C864, C52, and C1176 produce fragments of 45,000, 84,000, and 88,000

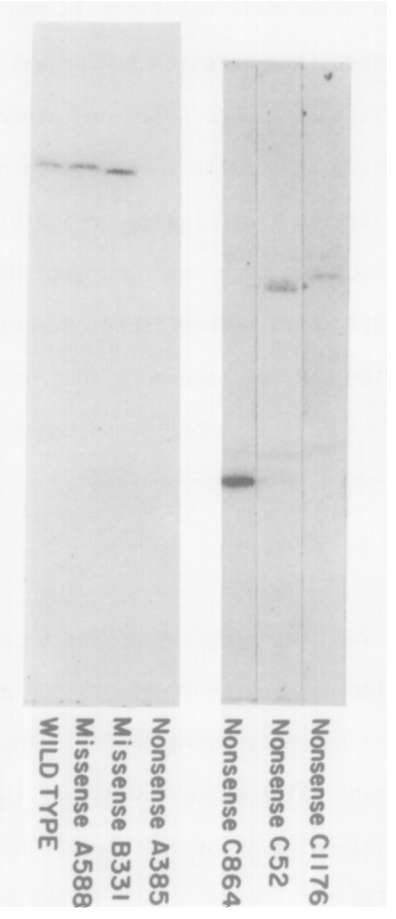


Figure 2. Direct identification of yeast wild-type and mutant HIS4 proteins by immuno-autoradiography on SDS-polyacrylamide gels. Crude extracts were subjected to electrophoresis on 10% polyacrylamide gels. After fixation gels were overlaid with antibody to the HIS4 protein, incubated, washed, and then reacted with radio-iodinated protein A. Each channel possesses a mutation designation. The gel on the left binds iodinated protein A only to 95,000 dalton bands, while the gel on the right reveals nonsense termination fragments.

daltons, respectively. Recently the HIS4 protein from a strain with mutation C864 has been purified and demonstrated to be 45,000 daltons (R. Bigelis and G.R. Fink, unpublished observations). The relative sizes of the three mutant proteins corresponds to the relative positions of the nonsense mutations on the genetic map (Figure 1). Using this technique we have also recently detected fragments generated by deletions

in the HIS4A region and by frameshift mutations in the HIS4C region. The use of a high ionic strength extraction buffer was critical in detecting some of these fragments (e.g. C1176 and others not shown). The high ionic strength could affect the conformation of the fragments, reducing their susceptibility to proteolysis, or inhibit the proteases themselves. A similar effect has been observed with HIS4C missense proteins (R. Bigelis and G.R. Fink, unpublished observations). Phenylmethylsulfonylfluoride did not have an effect on fragment stability. $(NH_4)_2SO_4$ and polyol effects on the stability of yeast proteins and on proteolysis have been discussed elsewhere (12-15).

## Discussion

The combined genetic and immunological approach confirms that the three HIS4 activities reside within the single 95,000 dalton protein and that the HIS4 region is not polycistronic (3). The identification and sizing of the HIS4 mutant polypeptides by direct immuno-autoradiography on SDS-gels now permits a correlation of the normal and mutant gene products of this region with the previous genetic map data. The genetic and polypeptide map (based on the size of the nonsense fragments) are colinear. Furthermore, the data shown in Figure 2 supports the earlier genetic determination of the direction of transcription of the HIS4 region as being from left to right since the further the nonsense mutation from the HIS4A region, the longer the nonsense fragment. Unlike conventional immunoprecipitation techniques the modified procedure we have used here to detect antigens directly in SDS-gels is particularly suited to work with unstable proteins such as mutant polypeptides. In this procedure immunological reaction has occurred after the proteins are resolved in the gel rather than before, and hence proteolytic degradation has been kept to a minimum allowing us to identify mutant proteins that were previously undetectable by other methods.

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