Gel Shift Assay of Nuclear Extracts from *Histoplasma capsulatum* Demonstrates the Presence of Several DNA Binding Proteins

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A gel shift assay was optimized to detect several general DNA binding proteins from *Histoplasma capsulatum* strain G217B. The electrophoretic mobility shift assay (EMSA) technique also detected protein(s) recognizing a pyrimidine-rich motif found in several *Histoplasma* promoters. Establishment of EMSA conditions provides an important framework to evaluate regulation of homeostatic or phase-specific genes that may influence virulence in *Histoplasma* and other dimorphic fungal pathogens.

*Histoplasma capsulatum*, a fungal pathogen prevalent in the Ohio and Mississippi River valleys, is the etiologic agent of histoplasmosis (3, 14). *H. capsulatum* is dimorphic, existing as a multicellular mycelium in the environment or at 25°C, and proliferating as a unicellular yeast within a host macrophage or at 37°C (9).

We have been interested in the control of general as well as phase-specific gene expression in *Histoplasma*, with YPS-3 regulation (4, 5) as a prototype. A pyrimidine-rich (TC) region in the YPS-3 promoter (1) exhibits phase-specific DNA binding activity in mycelial, but not in yeast phase, nuclear extracts in Southwestern blots from the virulent *H. capsulatum* strain G217B (1).

Electrophoretic mobility shift assays (EMSAs) have been problematic for many pathogenic fungi. In this study, we report optimized gel shift assay conditions with nuclear extracts from the mycelial and yeast phases of strain G217B.

**Extraction of nuclear proteins.** Mycelial and yeast phase cultures of strain G217B (ATCC 26032) were grown to mid-log phase at 25 or 37°C, respectively, and processed as previously described (1). The final protein pellet was resuspended in 10 mM Tris containing a protease inhibitor cocktail (1 g of aprotinin/ml, 0.3 g of leupeptin/ml, 0.3 g of benzamidine/ml, 0.55 g of aprotinin/ml, 0.1 g of pepstatin A/ml, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and stored in aliquots at −80°C until use.

**Oligonucleotides and labeling conditions.** The promoter regions of the YPS-3 gene (L16844) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AF273703) were scrutinized for consensus sequences of known transcription factors (Table 1). The following specific binding sites were used in this study: AP-1/C-JUN (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'), SP-1 (5'-ATTCGATCGGCTGCAGCTGGG-3'), TFIID (5'-ATTCGATCGGCTGCAGCTGGG-3'), NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3'), OCT-1 (5'-TGTCGAATGCAATCCTAGAA-3'), SP-1 (5'-ATTCGATCGGCTGCAGCTGGG-3'), and TC-1 (5'-ATTTCCTCTCCTTTTTCTTCTTT-3').

Double-stranded oligonucleotides (3 to 4 pmol) were end labeled with [γ-32P]dATP (3,000 Ci/mmol at 10 μCi/ml) and T4 polynucleotide kinase for 30 min at 37°C. The reaction was terminated with 1 μl of 0.5 M EDTA, diluted with Tris-EDTA, and purified (P-6 column [Bio-Rad]) in 10 mM Tris-HCl, pH 7.4.

**Gel shift reaction conditions.** Nuclear protein extracts were thawed and desalted on P-6 columns prior to use. Each reaction mixture was assembled on ice and consisted of 5 μg of protein extract, 0.5 μg of nonspecific carrier DNA [poly(dI-dC) · poly(dI-dC)] and 10,000 cpm of labeled oligonucleotide in binding buffer (40 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM MgCl2, 2% glycerol, and protease inhibitor cocktail lacking phenylmethylsulfonyl fluoride and dithiothreitol). The binding specificity was assessed with 100-fold excess cold specific or nonspecific oligonucleotide. For electrophoresis, 1 μl of gel loading buffer (25 mM Tris, pH 7.5, 4% glycerol [final]) was added and the entire reaction mixture was loaded onto a 4% polyacrylamide gel. Gels were run at a constant rate of 10 V/cm for 3 h at 4°C with a comb width of 0.5 cm.

**Optimization of gel shift assay conditions and electrophoresis.** Individual variables were examined that impacted the quality of the resolved protein-DNA complexes. The amount of nonspecific DNA carrier was titrated over a 0.2- to 2.0-μg concentration range by using 8 μg of mycelial phase nuclear protein extract with labeled CREB double-stranded oligonucleotide (Fig. 1A). The reaction specificity was assessed with either 1.75 pmol of TFIIID consensus sequence used as a non-specific binding competitor or 1.75 pmol of cold CREB motif as a specific competitor. Reaction parameters with 5 μg of extract and 0.5 μg of poly(dI-dC) maintained a ratio of extract to nonspecific carrier of 10 and minimized smearing (Fig. 1A).

*Histoplasma* yeast-phase nuclear extracts were examined over a range of concentrations in reactions (Fig. 1B) with a double-stranded oligonucleotide (5'-ATACAGGTTCTGATCAT CTCTCGATGTCGCGCGA-3') representing a region of the YPS-3 promoter (L16844) having homology with the Blastomy-
ces dermatitidis WI-1 (AAA91036) or BAD-1 (16) promoter region. The major band was successfully competed with specific oligonucleotide, suggesting the presence of at least one yeast phase-specific DNA binding protein in the nuclear extract (Fig. 1B) (data not shown).

Incubation of the reaction components at room temperature for 15 min completely eliminated the shifted signal (Fig. 2). However, reactions assembled on ice produced complexes that were successfully competed (Fig. 2) and were unchanged when reactions were incubated for 15 min or 2 h on ice (data not shown). This incubation temperature effect was observed with all oligonucleotides tested. Dephosphatases or nucleases are unlikely to be responsible, as unbound labeled probe is identical at both temperatures and EDTA in the binding reactions did not modify the results (data not shown). Moreover, the protein inhibitor cocktail mix did not affect the stability of the shifted complexes (data not shown). No obvious smearing below the shifted complex was observed. Thus, the loss of shifted complexes with these two DNA probes may be attributed to protein-probe thermal instability, and the biological significance of this system must be examined carefully.

Several critical variables emerged from the optimization analysis. Binding reaction results are reproducible only if performed on ice for as little as 15 min. Significantly, the use of a small comb width, which maintained sample concentration for electrophoresis, was crucial for complex integrity and resolution. Wider combs (1.0 cm or greater) led to a poor quality of shifted complexes or complete elimination of the shifted signal.

EMSA for general Histoplasma transcription factors. Little is known about the specific mechanisms by which the mycelial form of the organism responds to crucial host environmental cues. Histoplasma contains an AP-1 (c-jun)-like factor, which is competed successfully with an excess of cold consensus oligonucleotide (Fig. 3). With c-fos, AP-1 forms active transcription factors that contribute to the oxidative stress and metal resistance (6) response in Saccharomyces cerevisiae.

Levels of second messengers like cyclic AMP may fluctuate in the hyphal-to-yeast phase conversion in H. capsulatum (10, 18), and EMSA provides support for two cyclic AMP-responsive transcription factors, AP-2 (19) and CREB (15). A single,
well-defined shifted complex is observed in reactions with both the AP-2 (Fig. 3) and CREB (Fig. 2A) oligonucleotides.

Several ubiquitous transcription factors were also detected in *Histoplasma* nuclear extracts (Fig. 3), including OCT-1 (11) and TFIID (8). In contrast, no factor similar to NF-κB (7) was detected in *Histoplasma* nuclear extracts (data not shown).

**Gel shift assay conditions detect complex interactions in a region of the YPS-3 promoter.** Mycelial phase extracts were evaluated with a pyrimidine-rich TC-1 oligonucleotide to extend earlier Southwestern analysis, which defined a single interacting 30- to 35-kDa species (1). Two distinct, shifted complexes were observed by EMSA (Fig. 3). Both the upper and lower shifted bands are competed with cold specific TC-1 oligonucleotide as the competitor. While it is likely that one of the shifted bands corresponds to the p35 M-probe interaction (1), the second band may be due to heterologous protein-protein interactions that form under more permissive EMSA conditions.

In summary, nuclear extract preparations from both phases of *H. capsulatum* were optimized and used to devise a method for evaluating specific interactions between these proteins and specific DNA targets. Conditions were optimized with respect to a number of critical variables, notably incubation temperature and electrophoretic conditions. *Histoplasma* homologues of AP-1, AP-2, SP-1, OCT-1, CREB, and TFIID, but not of NF-κB, were observed. Furthermore, the gel shift conditions established here are permissive for the detection of numerous transcription factor interactions. Establishment of conditions for the gel shift assay and the demonstration of transcription factor homologues in *H. capsulatum* are significant developments in an increasing array of tools that may be crucial for evaluating transcriptional control in this important dimorphic pathogen as well as other medically relevant fungi.
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REFERENCES


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