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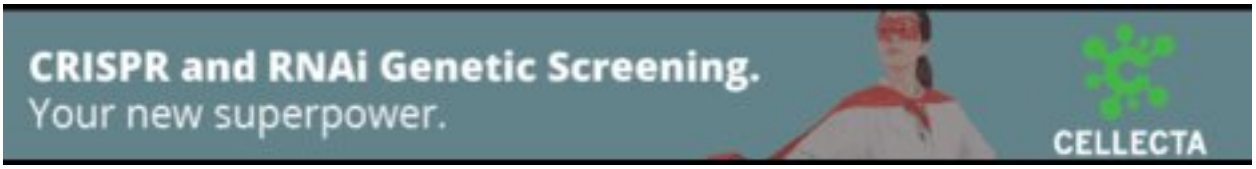

Letter

Temperature-Regulated Transcription in the Pathogenic Fungus *Cryptococcus neoformans*

Barbara R. Steen,[1] Tian Lian,[1] Scott Zuyderduyn,[2] William Kim MacDonald,[1] Marco Marra,[2] Steven J.M. Jones,[2] and James W. Kronstad[1,3]

[1]*Biotechnology Laboratory, Department of Microbiology and Immunology, and Faculty of Agricultural Sciences, The University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada;* [2]*Genome Sciences Centre, British Columbia Cancer Research Centre, Vancouver, British Columbia V5Z 4E6, Canada*

The basidiomycete fungus *Cryptococcus neoformans* is an opportunistic pathogen of worldwide importance that causes meningitis, leading to death in immunocompromised individuals. Unlike many basidiomycete fungi, *C. neoformans* is thermotolerant, and its ability to grow at 37°C is considered to be a virulence factor. We used serial analysis of gene expression (SAGE) to characterize the transcriptomes of *C. neoformans* strains that represent two varieties with different polysaccharide capsule serotypes. These include a serotype D strain of the *C. neoformans* variety *neoformans* and a serotype A strain of variety *grubii*. In this report, we describe the construction and characterization of SAGE libraries from each strain grown at 25°C and 37°C. The SAGE data reveal transcriptome differences between the two strains, even at this early stage of analysis, and identify sets of genes with higher transcript levels at 25°C or 37°C. Notably, growth at the lower temperature increased transcript levels for histone genes, indicating a general influence of temperature on chromatin structure. At 37°C, we noted elevated transcript levels for several genes encoding heat shock proteins and translation machinery. Some of these genes may play a role in temperature-regulated phenotypes in *C. neoformans*, such as the adaptation of the fungus to growth in the host and the dimorphic transition between budding and filamentous growth. Overall, this work provides the most comprehensive gene expression data available for *C. neoformans*; this information will be a critical resource both for gene discovery and genome annotation in this pathogen.

[This paper is dedicated to the memory of Michael Smith, founding director of the Biotechnology Laboratory and the British Columbia Genome Sciences Centre. The following individuals kindly provided reagents, samples, or unpublished information as indicated in the paper: Brendan Loftus, Claire Fraser, Richard Hyman, Eula Fung, Don Rowley, Ron Davis , Bruce A. Roe, Doris Kupfer, Jennifer Lewis, Sola Yu, Kent Buchanan, Dave Dyer, and Juneann Murphy.]

Cryptococcus neoformans has received considerable attention recently because of the high incidence of infections caused by this fungus in immunocompromised individuals (Casadevall and Perfect 1998; Harrison 2000). *C. neoformans* causes life-threatening infections in AIDS patients and people receiving immunosuppressive therapy. Cryptococcal meningitis is recognized as an AIDS-related infection, and *C. neoformans* is also capable of causing disease in immunocompetent individuals (Harrison 2000). Documented virulence factors include the production of a polysaccharide capsule, the formation of melanin, and the ability to grow at 37°C (Casadevall and Perfect 1998). Capsule-defective mutants of *C. neoformans* have reduced virulence compared with that of wild-type strains (Chang and Kwon-Chung 1998). Similarly, mutants defective in their ability to produce melanin on media containing phenolic compounds and mutants defective in their ability to grow at 37°C also show reduced virulence (Kwon-Chung and Rhodes 1986; Wang et al. 1995; Odom et al. 1997; Nosanchuk et al. 2000). The tolerance of *C. neoformans* to elevated temperatures has not been explored in detail, although it is known that mutations in *RAS1* and in *CNA1* (encoding calcineurin) cause growth defects at elevated temperature (Odom et al. 1997; Alspaugh et al. 2000). There is also an intriguing connection between mating and virulence in *C. neoformans*; strains of mating-type MATα have been shown to be more virulent than strains of the MAT**a** mating type, and the majority of clinical isolates are MATα (Kwon-Chung et al. 1992). One explanation for this prevalence is that only strains of the MATα mating type form the filamentous cell type that produces the small spores believed to serve as infectious propagules (Wickes et al. 1996).

C. neoformans is a dimorphic fungus that displays a yeast morphology in the haploid phase of the life cycle and a filamentous, dikaryotic cell morphology on mating between compatible haploid strains (Kwon-Chung and Bennett 1992; Casadevall and Perfect 1998). *C. neoformans* grows primarily by budding during infection, although filamentous growth is sometimes observed in the host (Bemis et al. 2000). Haploid strains of the MATα mating type can also show filamentous growth in culture in response to nitrogen starvation (Wickes et al. 1996). This filamentous growth (termed haploid fruiting) is associated with the formation of small asexual spores, which may serve as infectious agents via inhalation. Recently, it has been shown that stable diploid strains of *C. neoformans* can be obtained from crosses of compatible haploid mating

[3]Corresponding author.
E-MAIL kronstad@interchange.ubc.ca; FAX (604) 822-6097.
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partners (Sia et al. 2000). These diploid strains are thermally dimorphic in that they grow as yeast at 37°C and have a filamentous morphology at 24°C. At the lower temperature, the filaments formed by the diploid strains sporulate to produce haploid, meiotic progeny. Temperature regulation of the morphological switch in *C. neoformans* is reminiscent of the situation in other fungal pathogens of humans, including *Histoplasma capsulatum, Blastomyces dermatitidis*, and *Paracoccidioides brasiliensis* (Medoff et al. 1987; Maresca et al. 1994).

An international consortium has been established to determine the genomic sequence of *C. neoformans* (Heitman et al. 1999b). Initially, the MATα strain JEC21 was chosen for sequencing because this strain and a congenic MAT**a** isolate (JEC20) have been developed as genetically useful experimental strains (Heitman et al. 1999a). These strains represent the *neoformans* variety of *C. neoformans* defined in part by the D serotype of the polysaccharide capsule. In addition, there is considerable interest in obtaining the genomic sequence of other varieties of *C. neoformans*, including the clinical isolate H99 of the serotype A group of *C. neoformans* (variety *grubii*). A genomic shotgun sequencing effort is underway at Stanford University and at The Institute for Genomic Research (TIGR) for serotype D strain JEC21 and a related (progenitor) strain, B3501 (Heitman et al. 1999a). Expressed sequence tag (EST) projects for strains JEC21 and H99 are ongoing at the University of Oklahoma's Advanced Center for Genome Technology. In addition, limited shotgun sequencing has been performed for H99 at the Duke University Center for Genome Technology. To contribute to sequencing efforts, we have constructed physical maps of the genomes of strains JEC21 and H99 by bacterial artificial chromosome (BAC) fingerprinting, and we have performed BAC end sequencing to contribute to assembly of the genomic sequences (J. Schein et al. 2002).

In this report, we describe the use of serial analysis of gene expression (SAGE) to examine the transcriptome of *C. neoformans* as a function of temperature. SAGE involves generating short sequence (nine to 13 bp) tags that represent individual transcripts and using large-scale sequencing to establish the frequency of occurrence of these tags as a measure of transcript levels (Velculescu et al. 1995). SAGE has been used to define the transcriptome for *Saccharomyces cerevisiae* (Velculescu et al. 1997) and to explore transcription in normal and tumor cells (see Zhang et al. 1997). We chose SAGE instead of microarrays for defining the *C. neoformans* transcriptome because the small collections of available ESTs precluded the use of microarrays. In addition, SAGE data are digital and provide the opportunity for robust statistical analysis (Audic and Claverie 1997). Furthermore, when used in conjunction with genomic sequence data, SAGE results have been useful in all stages of genome annotation and, in particular, for gene identification (see Jones et al. 2001). Our experiments show the utility of SAGE for the genome-wide analysis of transcription in fungi and represent the first application of this technique to a human pathogen. Our SAGE analysis for *C. neoformans* revealed substantial differences in the transcriptomes of different serotypes and allowed the identification of sets of genes whose transcript levels vary with temperature. The characterization of the latter genes provides insight into the ability of *C. neoformans* to grow at 37°C in the human host.

RESULTS AND DISCUSSION

Temperature Regulation of Transcript Levels in *C. neoformans*

Four SAGE libraries were constructed and sequenced to generate RNA expression data for *C. neoformans* strains B3501 and H99, each grown at 25°C and 37°C. A summary of the collection of tags for each library is presented in Table 1. The collection and processing of the tag data included the use of `Phred` scores for the sequence traces to establish a statistical level of confidence in the sequence of each tag (see Methods). The data shown in Table 1 reflect `Phred` scores that provide a 99% probability that each tag sequence is correct. The collection of SAGE tags at two different temperatures provided a means to assess genome-wide changes in expression for two strains. Figure 1 presents the expression profile at the two temperatures for the serotype A strain H99. Of 12,056 tag species analyzed, 12.5% (1507 tag species) showed a significant difference ($P \leq 0.05$) between the two temperatures. A tag species is defined as the unique sequence identifier of a particular tag. Figure 2 presents the expression profile at the two temperatures for the serotype D strain B3501. For this strain, a total of 13,615 tag species were analyzed, and 4.9% (664 tag species) showed a significant difference ($P \leq 0.05$) between the two temperatures. For comparison, a recent analysis of the influence of temperature on global gene expression in group A *Streptococcus* revealed that 9% of the genes were differentially transcribed at 29°C versus 37°C (Smoot et al. 2001).

Identification of Genes for the Most Highly Expressed Tags for the Serotype A and D Strains

Although an annotated genomic sequence is not available for any strain of *C. neoformans*, we were able to make preliminary tag assignments to specific predicted genes with the partial genomic and EST sequence data for both strains. For strain H99, we have made preliminary gene assignments for 19 and 29 of the top 50 most abundant tags from the 25°C and 37°C libraries, respectively (Table 2A,B). In this strain, 20 tags were found to be identical in the top 50 of both libraries. A total of 70 unique tag species were studied, and 42 of these were associated with an EST sequence; 38 of the EST sequences gave significant `BLASTP` results, leading to putative gene assignments. Within the top 50 tags,

Table 1. Analysis of SAGE libraries

	H99		B3501	
	25°C	37°C	25°C	37°C
Sequence reads	1815	2213	4126	2165
Total tags*	30,181	37,467	65,399	15,363
Tag families				
Singletons	4406 (14.1%)	4196 (10.6%)	5967 (8.8%)	3693 (23.8%)
2 to 10	2703 (8.7%)	2821 (6.9%)	4468 (6.6%)	2029 (13.0%)
11 to 100	441 (1.4%)	564 (1.4%)	1082 (1.6%)	201 (1.3%)
>100	25 (0.09%)	43 (0.13%)	65 (0.11%)	3 (0.02%)
Total	7575	7624	11,582	5926

*Ninety-nine percent probability that each tag sequence is correct.

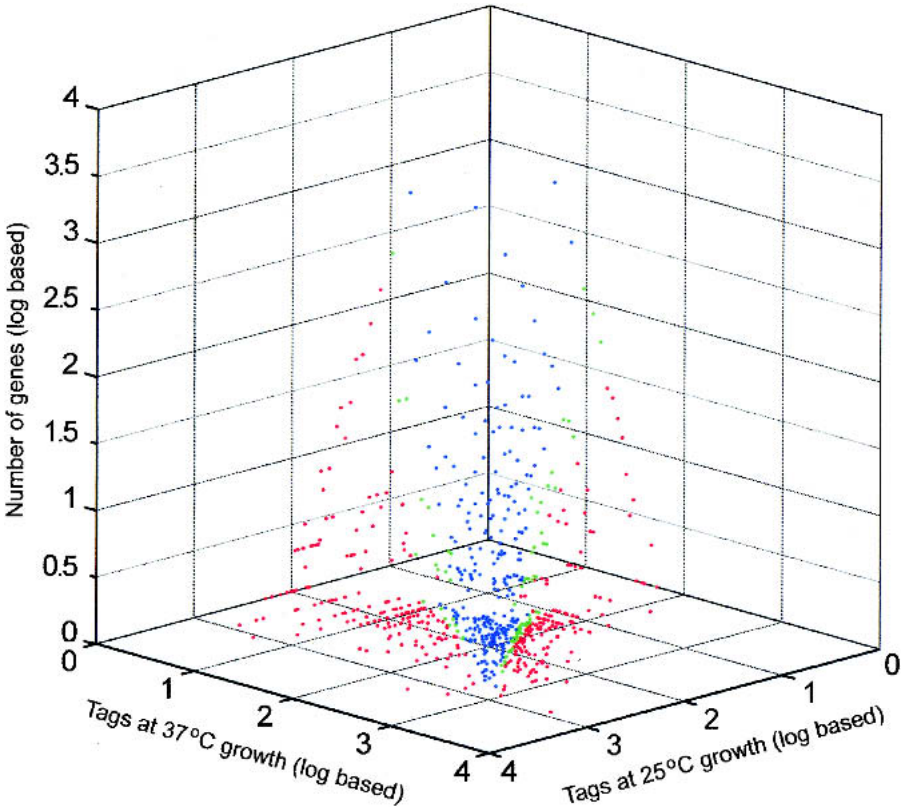

Figure 1 Expression profile comparing relative transcript levels at 25°C and 37°C in strain H99. Singleton tags were excluded. Blue dots indicate tags that do not show a significant expression difference; green dots, tags with a difference that is significant at 95% to 99% confidence; and red dots, tags with a significance of >99% confidence.

we identified genes for three ribosomal proteins at 25°C and 12 ribosomal proteins at 37°C. Furthermore, the top 50 tags (for both libraries) identified genes for proteins that are generally considered to be abundant in other organisms. These include GAPDH, translation elongation factor, pyruvate decarboxylase, malate dehydrogenase, and fructose-bisphosphate aldolase. Interestingly, a tag representing the transcript of a zinc transport protein was the most highly expressed tag at 25°C but was not seen in the top 50 tags for the 37°C library. As well, the tag representing cyclophilin A (*CPA1* and *CPA2*) was identified in the top 50 of both libraries but was expressed 1.47 times higher at 37°C. The genes encoding cyclophilin A have been characterized in *C. neoformans*, and Cpa1 is required for growth at elevated temperature and for virulence (Wang et al. 2001). Two of the abundant tags at 37°C identified transcripts for a thioredoxin peroxidase (0.74%) and a superoxide dismutase (0.35%). These tags were approximately fourfold higher at 37°C relative to 25°C. Lee and Park (1998) have shown that a thioredoxin peroxidase contributes to thermotolerance in *S. cerevisiae,* presumably by acting as an antioxidant. Superoxide dismutase plays a well-characterized role in antioxidant defense, and the production of the enzyme is known to be higher at 37°C than at 25°C in *C. neoformans* (Jacobson et al. 1994). The expression of this protein is also known to be influenced by temperature in other pathogens such as group A *Streptococcus* (Smoot et al. 2001). In general, these results indicate that growth at 37°C may induce the expression of genes involved in a stress response in *C. neoformans*.

The availability of more genomic sequence information for the serotype D strains JEC21 and B3501 (relative to strain H99) allowed us to make preliminary gene assignments for 33 and 34 of the top 50 most abundant tags prepared with cells grown at 25°C and 37°C, respectively (Table 3A,B). In this strain, substantially more tags (33) were found to be identical in the top 50 of both libraries compared with the H99 libraries. This finding is consistent with the lower percentage of differentially expressed genes for B3501 (Fig. 2). In total, 141 unique tag species were studied for strain B3501, 75 of which were given putative gene assignments based on a significant BLASTP result. Only eight tags did not associate with an EST or a genomic sequence contig. Of those tags that did not result in a putative gene assignment, 20 tags were ambiguous because they hit more than one sequence contig.

As in the H99 libraries, the top 50 tags represented genes for four (25°C) and 11 (37°C) ribosomal proteins, as well as genes for proteins that are expected to be abundant such as translation elongation factor, pyruvate decarboxylase, and GAPDH. The tag for the cyclophilin A transcript was seen at both 25°C and 37°C for B3501, although the tag was differentially expressed in an opposite manner (approximately twofold higher at 25°C in B3501) compared with the results for the H99 libraries. Also in contrast to the H99 libraries, the 50 most abundant tags in both B3501 libraries did not include a tag representing fructose-bisphosphate aldolase. For B3501, the list of abundant tags also revealed high transcript levels for the genes predicted to encode a ubiquitin RPS27A fusion protein, a ubiquitin conjugating enzyme, an iron permease, and a serine-threonine protein kinase that may be involved in pre-mRNA splicing (similar to Prp4p of *Schizosaccharomyces pombe*; Schwelnus et al. 2001). These genes were not identified in the top 50 tags from the H99 libraries at either temperature. In addition, the most abundantly expressed genes from both the 25°C and 37°C libraries of B3501 contained a zinc transporter that was seen only in the 25°C library from H99.

The B3501 37°C library revealed tags representing several proteins not seen in the 25°C library. These included the ER chaperone BiP (approximately twofold higher at 37°C), a peripheral benzodiazepine receptor homolog (discussed below), and several ribosomal proteins. Interestingly, the thioredoxin peroxidase tag that was found only in the top 50 tags of the 37°C library from H99 was identified in both the 25°C and 37°C top 50 tags of strain B3501. Overall, these results indicate that there are several differences in the response of H99 and B3501 to elevated temperature. A more extensive comparison will be possible when more tags can be matched with genes on completion and annotation of the genomic sequences of both strains.

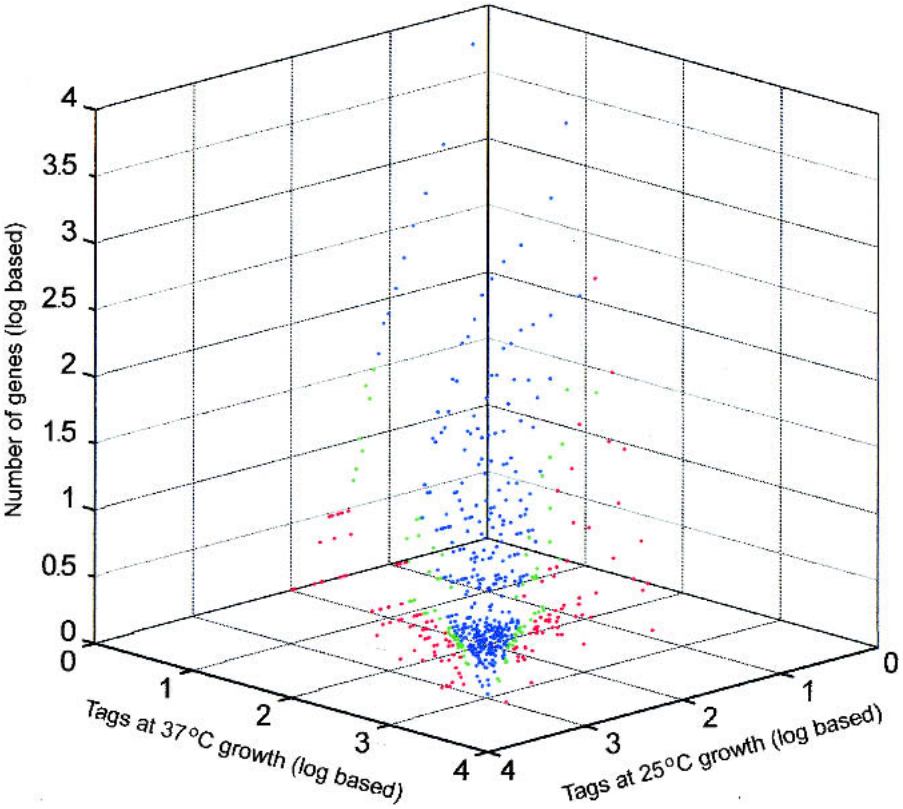

Figure 2 Expression profile comparing relative transcript levels at 25°C and 37°C in strain B3501. Singleton tags were excluded. Blue dots indicate tags that do not show a significant expression difference; green dots, tags with a difference that is significant at 95% to 99% confidence; and red dots, tags with a significance of >99% confidence.

The SAGE analysis of the most highly expressed genes in *C. neoformans* is comparable to that of *S. cerevisiae* (Velculescu et al. 1997). In yeast, the proteins encoded by the top 30 highly expressed genes included GAPDH, translation elongation factor-α, alcohol dehydrogenase, fructose-bisphosphate aldolase, pyruvate decarboxylase, and 18 ribosomal proteins. On the other hand, a comparison of our results with the changes in transcript levels observed for *S. cerevisiae* genes at 25°C and 37°C (as measured by microarray analysis; Gasch et al. 2000) indicates that temperature influences the transcription of a relatively greater number of genes in *C. neoformans*.

Tags With Higher Levels at 25°C

To begin to determine differences in transcript levels at the two temperatures, we made preliminary gene assignments for a selected group of 100 tags that showed the most statistically significant different expression levels between the two temperatures. All of these tags have a value of $P < 0.05$ as the minimum level of significance for concluding that a given tag showed differential expression. The fold difference for the tag levels was determined by normalizing the total tag numbers to represent libraries of equal sizes. We note that the calculation of fold-difference is less accurate in this analysis when the number of tags is small, although the *P* value calculation is unaffected. We focused our analysis on the data for strain B3501 because, as noted earlier, there is substantially more genomic sequence information available for this strain compared with H99.

The analysis of 50 tags with higher levels at 25°C revealed several patterns of transcription that may reflect general features of temperature adaptation in *C. neoformans* (Table 4). First, the tags representing transcripts for histones H1, H3, and H4 were all elevated at 25°C compared with 37°C (approximately two- to sevenfold). Assuming that these changes in transcript levels reflect changes in the abundance of histone proteins, our results indicate that growth temperature may exert a general influence on chromatin structure in *C. neoformans*. This was corroborated by the fact that at 37°C H4 was expressed 10-fold more than H1, whereas at 25°C, H4 was expressed only threefold more than H1. These observations indicate that growth temperature causes a change in the relative expression of histone gene families. In turn, this may reflect a broad shift in gene expression for this pathogen as a function of temperature. This conclusion is supported by results from *S. cerevisiae* in which the examination of changes in histone abundance (e.g., by depletion of histone H4) revealed changes in the expression of ~25% of all of the genes (Wyrick et al. 1999).

A second notable group of tags that were up-regulated at 25°C represented genes for sterol and lipid metabolism. The expression pattern for these genes is consistent with observations in other organisms in which adjustments in membrane composition are correlated with growth temperature (Steels et al. 1994; Los et al. 1997; Aguilar et al. 1998). In general, cells adapt to a lower temperature by an increase in the production of desaturase, resulting in unsaturated fatty acids in membrane phospholipids to maintain proper fluidity. That is, we would expect the SAGE data to reveal changes in transcript levels for desaturase genes as a function of temperature, and we did identify a tag for the transcript of a Δ9 fatty acid desaturase that was elevated 14.56-fold at 25°C. Other tags that were elevated at 25°C included those representing genes for sterol synthesis (sterol C-5 desaturase and C-4 methyl sterol oxidase) and fatty acid synthesis (fatty acid synthases). Sterol content in *C. neoformans* is known to change in response to passage of the fungus through an animal host (Currie et al. 1995). Changes in membrane composition have also been correlated with morphogenesis and thermotolerance in other fungal pathogens. For example, a Δ9 fatty acid desaturase is regulated by temperature and cAMP signaling during the dimorphic transition in *Histoplasma capsulatum* (Storlazzi et al. 1999). These observations may be relevant for *C. neoformans* because signaling via a cAMP pathway is known to play an important role in the virulence (Alspaugh et al. 1997, 2001; D'Souza et al. 2001).

Table 2A. Top 50 Tags Expressed at 25°C for Strain H99

SAGE tag	Frequency (30,181 total)	% Abundance	Prelimary gene designation	E-value of BLASTx	Accession no. of BLASTx
ttcagcaggc	430	1.42%	Zinc transport protein	7.00E-15	*Saccharomyces cerevisiae* Z72777
ctcagcgatg	352	1.17%	NO HIT[a]		
cattcgcata	309	1.02%	NO HIT[a]		
cgacagaccg	222	0.74%	Translation elongation factor 1α	0.00E + 00	*Cryptococcus neoformans* U81804
aaaaaaaaaa	211	0.70%	NO HIT[a]		
atatgacata	210	0.70%	NO HIT[a]		
gccaacgccg	203	0.67%	Cyclophilin A	2E-72[c]	*C. neoformans* U81804
gctctccagg	171	0.57%	NO HIT[a]		
catctgttcc	171	0.57%	NO HIT[a]		
cgcggaaagg	162	0.54%	NO HIT[a]		
tagcgatcac	153	0.51%	NO HIT[a]		
tagccgcgaa	153	0.51%	NO HIT[b]		
ataagctttc	148	0.49%	Mannitol 1-phosphate dehydrogenase	3.00E-18	*C. neoformans* AF175685
gtttccgctg	147	0.49%	NO HIT[a]		
ttcggcaagg	132	0.44%	ADP, ATP carrier protein	1.40E-131	*Neurospora crassa* X00363
gtcggtggta	130	0.43%	ATP synthase β-chain	8.00E-59	*Kluyveromyces lactis* U37764
gtggacacga	129	0.43%	Nucleoside diphosphate-sugar hydrolase	4.00E-26	*S. cerevisiae* CAA85068
aatgaatctt	122	0.40%	NO HIT[a]		
tctggtcgag	121	0.40%	Histone H4	2.90E-36	*Agaris bisporus* P35058
tcagaagttg	121	0.40%	Thioredoxin	9.00E-25	*Coprinus comatus* AJ242791
agcgagcact	120	0.40%	NO HIT[a]		
gtattgaccc	113	0.37%	Hypothetical protein	4E-69[c]	*Streptomyces coelicolor* AL132991
atgatcgggc	108	0.36%	NO HIT[a]		
aaaaacgcgt	107	0.35%	Myo-inositol-1-phosphate synthase	1.00E-66	*Drosophila melanogaster* AF071103
catcactctt	103	0.34%	Pyruvate decarboxylase	8.0E-34[c]	*Saccharomyces kluyveri* AF193853
ccgcgaccgt	98	0.32%	NO HIT[a]		
gctgcctaca	93	0.31%	ATP synthase—γ-chain	5.00E-21	*N. crassa* AL355930
acggtggcaa	92	0.30%	NO HIT[a]		
acacgtctgg	91	0.30%	NO HIT[a]		
ggttacgccg	91	0.30%	Malate dehydrogenase	2.00E-35	*S. cerevisiae* J02841
gcgttctcgg	86	0.28%	Transaldolase	1.00E-102	*Schizosaccharomyces pombe* AL023518
actcaggttg	83	0.28%	Fructose 1,6-bisphosphate aldolase	2.00E-46	*N. crassa* L42380
gaatagtggg	81	0.27%	NO HIT[a]		
ggccgacctg	80	0.27%	60S ribosomal protein RPL11	3.00E-81	*S. pombe* Z69240
atgcatttcg	80	0.27%	NO HIT[a]		
gctcgcgacg	77	0.26%	60S ribosomal protein RPL2	1.00E-103	*Xenopus laevis* U00920
atatgtatcg	75	0.25%	NO HIT[a]		
aacgtctgcc	74	0.25%	NO HIT[a]		
accgtcgttg	74	0.25%	NO HIT[a]		
tgcaaacgcg	74	0.25%	Peroxisomal membrane protein	6.00E-12	*S. pombe* AJ002536
gcgccgctta	72	0.24%	NO HIT[a]		
aagcgcatttt	71	0.24%	NO HIT[a]		
tagtgtcccg	70	0.23%	NO HIT[a]		
aagggtggtg	68	0.23%	NO HIT[a]		
aagcctgacg	67	0.22%	NO HIT[b]		
aaatggtttg	66	0.22%	NO HIT[a]		
catcacgctt	64	0.21%	60s ribosomal protein RPL5	3.00E-26	*S. pombe* AL031528
agcaaggagg	63	0.21%	NO HIT[a]		
taacgcataa	63	0.21%	NO HIT[b]		
agcaaggagg	63	0.21%	NO HIT[a]		

[a]Serial analysis of gene expression (SAGE) tag does not have an associated expressed sequence tag (EST) at http://www.genome.ou.edu/cneo.html.
[b]Identified EST does not have a significant BLASTx result at http://www.ncbi.nlm.nih.gov/.
[c]BLASTx results for a contig identified at http://mgm.duke.edu.

An additional general observation for the tags with higher levels at 25°C is that many represent genes for transport functions. These included a gene involved in iron transport, as well as glucose and inositol transporters. Inositol metabolism has been examined in *C. neoformans* and is proposed to be important for pathogenesis (Luberto et al. 2001). This may be relevant for virulence because of the preference of *C. neoformans* for growth in the central nervous system, a location known to be rich in inositol (Vincent and Klig 1995). We also found that the tag for a putative

Table 2B. Top 50 Tags Expressed at 37°C for Strain H99

SAGE tag	Frequency (37,467 total)	% Abundance	EST hit	E-value	Accession no. of BLASTx
cgacagaccg	931	2.48%	Translation elongation factor 1α	0.00E + 00	*C. neoformans* U81804
ggcctcggtt	387	1.03%	NO HITS[a]		
tccccgtaca	330	0.88%	NO HITS[a]		
gccaacgccg	300	0.80%	Cyclophilin A	2E-72[c]	*C. neoformans* U81804
cacgttcacg	276	0.74%	Thioredoxin peroxidase	9.00E-64	*S. pombe* AL031798
aacgtctgcc	272	0.73%	NO HITS[a]		
cgcggaaagg	264	0.70%	NO HITS[a]		
gctcgcgacg	259	0.69%	60S ribosomal protein RPL2	1E-103	*X. laevis* U00920
ggccgacctg	256	0.68%	60S ribosomal protein RPL11	3E-81	*S. pombe* Z69240
gtcggtggta	228	0.61%	ATP synthase β-chain	8E-59	*Kluyveromyces lactis* U37764
gtttccgctg	223	0.60%	NO HITS[a]		
aagggtggtg	204	0.54%	NO HITS[a]		
aagcccgttg	194	0.52%	NO HITS[a]		
tctgtcgagg	183	0.49%	40S ribosomal protein RPS12	3E-41	*Susscrofa* X79417
gagaagcgtg	174	0.46%	60S ribosomal protein RPL21A	4.10E-51	*S. cerevisiae* M86408
ctcagcgatg	173	0.46%	NO HITS[b]		
cacggcgcat	164	0.44%	60S ribosomal protein RPL41	2.00E-58	*Xanthophyllomyces dendrorhous* AF004672
taggccgtct	158	0.42%	NO HITS[a]		
aaggactctc	158	0.42%	40S ribosomal protein RPS15	2.60E-42	*Podospora anserina* Z23267
gctctccagg	155	0.41%	NO HITS[a]		
tctggtcgag	152	0.41%	Histone H4	2.9E-36	*Agaris bisporus* P35058
tccctattaa	151	0.40%	NO HITS[a]		
cagaaccccg	147	0.39%	40s ribosomal protein RPS18	6.9E + 45	*S. pombe* AL034564
acggccgtta	139	0.37%	NO HITS[a]		
aaaaaaaaaa	135	0.36%	NO HITS[a]		
ctcttcccct	135	0.36%	60S ribosomal protein RPL33B	9E-31	*S. cerevisiae* L23923
tctttccgag	135	0.36%	GAPDH	2.80E-59	*C. neoformans* AF106950
gtattgaccc	131	0.35%	Hypothetical protein	4.0E-69[c]	*Streptomyces coelicolor* AL132991
cacgtccacg	131	0.35%	Cu,Zn superoxide dismutase	5.5E-51	*Aspergillus fumigatus* AF128886
gctgcctaca	130	0.35%	ATP synthase—γ-chain	2.00E-33	*N. crassa* AL355930
gccgtccgaa	130	0.35%	40S ribosomal protein RPS5	4.40E-71	*Mus musculu* U78085
gctcctctta	128	0.34%	ATP synthase α-chain	1.90E-01	*N. crassa* M84191
tctttgatgt	125	0.33%	ADP, ATP carrier protein	1.4E-122	*N. crassa* X00363
tccatccgat	123	0.33%	60S ribosomal protein RPL10	8.90E-83	*S. cerevisiae* U06952
atgatcgggc	123	0.33%	NO HITS[a]		
gctttgctgc	122	0.33%	Hypothetical protein (Schizosaccharomyces pombe)	1.80E-11	*S. pombe* Z97992
atgggctccc	119	0.32%	ATP synthase—γ-chain	2.9E-30	*S. pombe* AL031856
gacgactcta	116	0.31%	NO HITS[a]		
gagttgttga	115	0.31%	60S ribosomal protein RPL36	3.6E-13	*S. pombe* D88771
actcaggttg	114	0.30%	Fructose 1,6-bisphosphate aldolase	2.0E-46[c]	*Aspergillus oryzae* AB032272
ccgcgaccgt	113	0.30%	NO HITS[a]		
gcttttgccc	110	0.29%	NO HITS[a]		
ttcggcaagg	107	0.29%	ADP, ATP carrier protein	1.4E-131	*N. crassa* X00363
tcggtcgtgt	104	0.28%	Suppressor protein STM1	0.05	*S. cerevisiae* D26183
cctcttcctg	102	0.27%	NO HITS[a]		
ggttacgccg	98	0.26%	Malate dehydrogenase	2E-35	*S. cerevisiae* Z28085
gcgttctcgg	95	0.25%	Transaldolase	3.30E-102	*S. pombe* AL023518
cgtgtcaagc	95	0.25%	NO HITS[a]		
gtcaagaagc	95	0.25%	NO HITS[a]		
ggtatcctcg	95	0.25%	Putative 40S ribosomal protein	5E-50	*S. pombe* NC_003424

[a]Serial analysis of gene expression (SAGE) tag does not have an associated expressed sequence tag (EST) at http://www.genome.ou.edu/cneo.html.
[b]Identified EST does not have a significant BLASTx result at http://www.ncbi.nlm.nih.gov/.
[c]BLASTx results for a contig identified at http://mgm.duke.edu

inositol synthase gene was up-regulated at 25°C, further indicating a connection between inositol metabolism and growth temperature.

In addition to our analysis of 50 differentially expressed tags (Table 4), we also found that the tag for a *C. neoformans* translation elongation factor-3 (TEF3; ATGTATATAC) was 6.10-fold more abundant at 25°C. TEF3 is a fungal-specific elongation factor, and transcript levels for this gene are known to change in *C. albicans* as a function of temperature. That is, changes in transcript levels have been observed during growth at different temperatures, although these changes do not seem to be associated with temperature-regulated dimorphism in this fungus. As well, there is evidence to support the idea that reduced transcription of TEF3 in *C. albicans* results in decreased virulence in a mouse model of infection (Nakayama et al. 2000).

Table 3A. Top 50 Tags Expressed at 25°C for Strain B3501

SAGE tag	Frequency (65,399 total)	Percentage	Preliminary gene designation	E-value of top BLASTx result	Accession no. of BLASTx
gaacgatgct	607	0.93%	NO HITS[b]		
catttacata	546	0.83%	NO HITS[b]		
cgagtcgtat	539	0.82%	Iron permease	2E-23	*Schizosaccharomyces pombe* Z67998
cgacagaccg	529	0.81%	Translation elongation factor 1	0.0/0.0[c]	*Cryptococcus neoformans* U81804
aaaaaaaaaa	452	0.69%	NO HITS[b]		
gtattgaccc	430	0.66%	Phosphoketolase	1.00E-165/1.00E-106[c]	*Lactococcus lactis* AE006381
aatgactttt	427	0.65%	NO HITS[b]		
gcgttacttg	348	0.53%	Zinc transporter	2E-27	*Saccharomyces cerevisiae* Z72777
tctttgatgt-3′	328	0.50%	ADP, ATP carrier protein	1.00E-110/2.00E-72[c]	*Gossypium hirsutum* AF006489
gtcgtagagt	327	0.50%	Enolase	1E-131	*S. cerevisiae* J01322
atatgacata	305	0.47%	Glycine dehydrogenase	0	*S. pombe* Z54308
caagtaattt	293	0.45%	NO HITS[b]		
catctattcc	286	0.44%	NO HITS[a]		
ccagaagttg	267	0.41%	Mitochondrial thioredoxin	2E-39/2.00E-54[c]	*S. cerevisiae* X59720
ttcggcaagg-5′	264	0.40%	ADP, ATP carrier protein	1.00E-115/1.00E-132[c]	*G. hirsutum* AF006489
ctccgccgag	261	0.40%	Pyruvate decarboxylase	1.00E-72/3.00E-40[c]	*Pichia stipitis* U75310
gctctccagg	250	0.38%	Histone H3	1.00E-48/9.00E-64[c]	*Mortierella alpina* AJ249812
gctaacgctg	238	0.36%	Cyclophilin A	5.00E-76/2.00E-91[c]	*C. neoformans* AF333996
gtcggtggta	230	0.35%	ATP synthase—β-chain	0.0/3.00E-43[c]	*Kluyveromyces lactis* U37764
tcgagaatgg	218	0.33%	NO HITS[b]		
gacgatatat	204	0.31%	C-4 methyl sterol oxidase	2E-84/2.00E-48[c]	*S. pombe* AL109832
cagagatgtg	197	0.30%	Nonhistone protein	1.00E-6/7.00E-9[c]	*S. cerevisiae* Z94864
tctggtcgag	187	0.29%	Histone H4	7.00E-19/2.00E-38	*Phanerochaete chrysosporium* Z15134
aggaagagaa	186	0.28%	Hypothetical protein	2.00E-22/5.00E-05[c]	*Agaricus bisporus* AJ271701
cgcggaaagg	184	0.28%	NO HITS[a]		
aaatggtttg	183	0.28%	NO HITS[b]		
tagccgggaa	182	0.28%	NO HITS[b]		
tccttccgag	179	0.27%	GAPDH	1.00E-112/0.0[c]	*C. neoformans* AF106950
atttccgccg	178	0.27%	Serine-threonine protein kinase	7E-65	*Mus musculus* U48737
cacgttcacg	168	0.26%	Thioredoxin peroxidase	2.00E-39/2.00E-64[c]	*S. pombe* AL031798
ataaaaaaaa	159	0.24%	NO HITS[a]		
catattgaat	157	0.24%	Uracil ribosyl transferase	3.00E-10	*S. pombe* Z98598
gcagatcgat	154	0.24%	60S ribosomal protein RPL39	3.00E-09/1.00E-13[c]	*K. marxianus* S53434
gctcctctta	152	0.23%	ATP synthase—α-chain	2.00E-58/9.00E-46[c]	*S. pombe* M57955
aaagcgcgtt	151	0.23%	Inositol 1-phosphate synthase	1E-144	*Pichia pastoris* AF078915
agtcctcttc	150	0.23%	60S ribosomal protein RPP2	1.00E-15	*Alternaria alternata* U87806
actaccttct	149	0.23%	Ribosomal protein RPP1	1E-13	*C. elegans* AF003139
ccatatgttt	149	0.23%	Glycogen phosphorylase	6.00E-95/2.00E-40[c]	*Dictyostelium discoideum* M77492
actatcgcct	142	0.22%	Ubiquitin conjugating enzyme	8.00E-45/2.00E-75[c]	*Glomerella cingulata* AF030296
cagcagttta	139	0.21%	NO HITS[b]		
agtggcagtt	138	0.21%	Opsin	0.004/3.00E-21[c]	*Leptosphaeria maculans* AF290180
cattcgttca	137	0.21%	NO HITS[b]		
aattcgcttt	133	0.20%	14-3-3 Protein	5.00E-84/1.00E-124[c]	*Schizophyllum commune* AY029473
tagcctttcg	127	0.19%	NO HITS[b]		
cgtgaggctg	125	0.19%	6-Phosphogluconate dehydrogenase	1.00E-170/0.0[c]	
catacaggtc	122	0.19%	Glutamine synthase	1.00E-133/1.00E-163[c]	*A. bisporus* Y12704
ggttacgctg	121	0.19%	Mitochondrial malate dehydrogenase	1.00E-115[c]	*S. cerevisiae* J02841
taacgcataa	117	0.18%	NO HITS[b]		
ccggctaatg	117	0.18%	NO HITS[b]		
acatcgatct	117	0.18%	60S ribosomal protein RPL31	3E-25	*Cyanophora paradoxa* AJ005204

[a]Serial analysis of gene expression (SAGE) tag does not have an associated genomic contig at Stanford or expressed sequence tag (EST) at http://www.genome.ou.edu/cneo.html.
[b]Identified EST or contig does not have a significant BLASTx result at http://www.ncbi.nlm.nih.gov/.
[c]EST BLASTx result.

Table 3B. Top 50 Tags Expressed at 37°C for Strain B3501

SAGE tag	Frequency (15,363 total)	Percentage	Preliminary gene designation	E-value of top BLASTx result	Accession no. of BLASTx
cgacagaccg	207	1.35%	Translation elongation factor 1	0.0/0.0[c]	*C. neoformans* U81804
aggaagagaa	125	0.81%	Hypothetical protein (Agaricus bisporus)	2.00E-22/5.00E-05[c]	*A. bisporus* AJ271701
aaaaaaaaaa	119	0.77%	NO HITS[b]		
gcgttacttg	85	0.55%	Zinc transporter	2.00E-27	*S. cerevisiae* Z72777
ctccgccgag	85	0.55%	Pyruvate decarboxylase	1.00E-72/3.00E-40[c]	*Pichia stipitis* U75310
gtcgtagagt	82	0.53%	Enolase	1E-131	*S. cerevisiae* J01322
gtcggtggta	76	0.49%	ATP synthase—β chain	0.0/3.00E-43[c]	*K. lactis* U37764
ccagaagttg	68	0.44%	Mitochondrial thioredoxin	2.00E-39/2.00E-72[c]	*S. cerevisiae* X59720
aatgacttttt	68	0.44%	NO HITS[b]		
atatgacata	67	0.44%	Glycine dehydrogenase	0	*S. pombe* Z54308
cgagtcgtat	62	0.40%	Iron permease	2E-23	*S. pombe* Z67998
catttacata	57	0.37%	NO HITS[b]		
ttcggcaagg-5′	57	0.37%	ADP, ATP carrier protein	1E-115/1.00E-132[c]	*G. hirsutum* AF006489
gaacgatgct	56	0.36%	NO HITS[b]		
atatgaaaga	55	0.36%	NO HITS[b]		
cgcggaaagg	54	0.35%	NO HITS[a]		
gtattgaccc	52	0.34%	Phosphoketolase	1.00E-165/1.00E-106[c]	*Lactococcus lactis* AE006381
tctttgatgt-3′	49	0.32%	ADP, ATP carrier protein	1.00E-110/2.00E-72[c]	*Gossypium hirsutum* AF006489
aacgtctgcc	45	0.29%	NO HITS[a]		
attgagatgg	44	0.29%	NO HITS[b]		
atttccgccg	43	0.28%	Serine-threonine protein kinase	7E-65	*M. musculus* U48737
actaccttct	42	0.27%	Ribosomal protein RPP1	1.00E-13	*C. elegans* AF003139
acgtaccttt	41	0.27%	NO HITS[b]		
cacaatcctt	41	0.27%	Ubiquitin/ribosomal protein RPS27A fusion protein	6.00E-36/7.00E-40[c]	*N. crassa* U01220
ggccgacctg	41	0.27%	Ribosomal protein RPL11	2.00E-56/5.00E-73[c]	*S. pombe* Z69240
catctattcc	40	0.26%	NO HITS[a]		
cacgttcacg	40	0.26%	Thioredoxin peroxidase	2.00E-39/2.00E-64[c]	*S. pombe* AL031798
gcattggcgt	39	0.25%	ER chaperone BiP	0.0/5.00E-20	*Aspergillus oryzae* AB030231
actatcgcct	38	0.25%	Ubiquitin conjugating enzyme	8.00E-45/2.00E-75[c]	*Glomerella cingulata* AF030296
gctcgcgacg	36	0.23%	60S ribosomal protein RPL2	2.00E-72	*D. melanogaster* AF098520
tccttccgag	36	0.23%	Glyceraldehyde-3-phosphate dehydrogenase	1.00E-112/0.0[c]	*C. neoformans* AF106950
cctgttctcg	36	0.23%	NO HIT[b]		
tctgtcgagg	35	0.23%	40S ribosomal protein RPS12	6.00E-36/7.00E-42[c]	*S. pombe* AL031154
cattcgttca	35	0.23%	NO HIT[b]		
tagcctttcg	34	0.22%	NO HIT[b]		
atgggctccc	34	0.22%	ATP synthase—γ-chain	6.00E-44/9.00E-66[c]	*S. pombe* AL031856
gctcctctta	33	0.21%	ATP synthase—α-chain	2.00E-58/9.00E-46[c]	*S. pombe* M57955
acatcgatct	32	0.21%	60S ribosomal protein RPL31	3E-25	*Cyanophora paradoxa* AJ005204
gcagatcgat	32	0.21%	60S ribosomal protein RPL39	3.00E-09/1.00E-13[c]	*K. marxianus* S53434
gatgcttttt	30	0.20%	60S ribosomal protein RPL19	9.00E-31	*S. pombe* AB010048
ggttacgctg	30	0.20%	Mitochondrial malate dehydrogenase	1.00E-115[c]	*S. cerevisiae* J02841
cggtgcctgc	30	0.20%	60S ribosomal protein RPL15	3.00E-47/4.00E-88[c]	*Quercus suber* AJ001346
aaatggtttg	29	0.19%	NO HIT[b]		
gctaacgctg	29	0.19%	Cyclophilin A	5.00E-76/2.00E-91[c]	*C. neoformans* AF333996
aaccgcacca	29	0.19%	Peripheral benzodiazepine receptor	6.00E-16[c]	*Homo sapiens* JE0149
agtcctcttc	28	0.18%	60S ribosomal protein RPP2	1E-15	*Alternaria alternata* U87806
cagcagttta	28	0.18%	NO HITS[b]		
cacggcgcat	27	0.18%	60S ribosomal protein RPL41	2E-39	*C. neoformans* AF118148
tagccgggaa	27	0.18%	NO HITS[b]		
catagttggt	27	0.18%	Heat shock protein 70 family	0	*Malassezia sympodialis* AJ428052

[a]Serial analysis of gene expression (SAGE) tag does not have an associated contig at Stanford or expressed sequence tag (EST) at http://www.genome.ou.edu/cneo.html.
[b]Identified EST does not have a significant BLASTx result at http://www.ncbi.nlm.nih.gov/.
[c]EST BLASTx result.

Table 4. B3501 Tags More Highly Expressed at 25°C

SAGE tag	B3501 25	25 normalized	B3501 37	FOLD difference	Preliminary gene designation	E-value: Genomic BLASTx	E-value: EST BLASTx	Accession no. of BLASTx
caagtaattt	293	69	11	6.3	NO HITS[b]			
gaacgatgct	607	143	56	2.6	NO HITS[b]			
aaagcgcgtt	151	35	3	11.7	Inositol 1-phosphate synthase	1.00E-144		*Pichia pastoris* AF078915
catttacata	546	128	57	2.2	NO HITS[b]			
gacgatatat	204	48	9	5.3	C-4 methyl sterol oxidase	2.00E-84	2.00E-48	*Schizosaccharomyces pombe* AL109832
gctctccagg	250	59	15	3.9	Histone H3	2.00E-48	9.00E-64	*Mortierella alpina* AJ249812
cgagtcgtat	539	127	62	2.0	Iron permease	2.00E-23		*S. pombe* Z67998
cagagatgtg	197	46	14	3.3	Nonhistone protein	1.00E-06	7.00E-09	*Saccharomyces cerevisiae* Z94864
tatctgaaag	93	22	2	11.0	Delayed-type hypersensitivity antigen	1.00E-103		*Cryptococcus neoformans* AF246128
cattcggttt	60	14	0	unique to 25	NO HITS[b]			
gtattgaccc	430	101	52	1.9	Phosphoketolase	1.00E-165	1.00E-106	*Lactococcus lactis* AE006381
tgatgggaag	57	13	0	unique to 25	Sterol C-5-desaturase	1.00E-56		*Rattus norvegicus* AB052846
tcgagaatgg	218	51	19	2.7	NO HITS[b]			
aattcgcttt	133	31	8	3.9	14-3-3-Protein	5.00E-84	1.00E-124	*Schizophyllum commune* AY029473
tatatgtgta	79	19	2	9.5	Heat shock protein 12	7.50E-02	4.00E-12	*S. cerevisiae* X55785
ataaaaaaaa	159	37	12	3.1	NO HITS[a]			
tgaaaatata	62	15	1	15.0	Δ9 fatty acid desaturase	1.00E-136	1.00E-111	*Mortierella alpina* Y18553
tacttttttt	108	25	6	4.2	NO HITS[a]			
tatcccacca	99	23	5	4.6	NO HITS[b]			
atgatttgag	77	18	3	6.0	NO HITS[b]			
cctcaacggc	101	24	6	4.0	NO HITS[b]			
gaactggcgg-3′	37	9	0	unique to 25	Adenosyl homocysteinase	0.00E + 00	2.00E-40	*S. pombe* AB004537
agtgctgctg	63	15	2	7.5	Histone H1	1.10E-02		*S. cerevisiae* U43703
caaaaaggat	63	15	2	7.5	NO HITS[b]			
tcaaagaaga	62	15	2	7.5	NO HITS[b]			
ctgaggctga	50	12	1	12.0	NO HITS[b]			
ggcttgacca	50	12	1	12.0	High-affinity monosaccharide transporter	9.00E-30	8.00E-48	*Amanita muscaria* Z83828
ccggctaatg	117	27	9	3.0	NO HITS[b]			
ctgtatgtcc	34	8	0	unique to 25	NO HITS[b]			
accttgatgg	78	18	4	4.5	NO HITS[b]			
gacttttgac	94	22	6	3.7	NO HITS[b]			
tctggtcgag	187	44	20	2.2	Histone H4	7.00E-19	2.00E-38	*Agaris bisporus* P35058
ggcatttagt	32	8	0	unique to 25	NO HITS[b]			
attggtttga	32	8	0	unique to 25	NO HITS[b]			
gctaacgctg	238	56	29	1.9	Cyclophilin A	5.00E-76	2.00E-91	*C. neoformans* AF333996
ttcgcgctaa	66	16	3	5.3	NO HITS[b]			
caagcagata	45	11	1	11.0	Fatty acid synthase α-chain	1.00E-68		*S. pombe* D83412
tatccgggtc	72	17	4	4.3	Aspartate aminotransferase	6.00E-89		*Homo sapiens* M22632
tctaacccta	28	7	0	unique to 25	Hmp1 of U. maydis	1.00E-14	1.00E-22	*Ustilago maydis* U39049
cacattgata	70	16	4	4.0	NO HITS[b]			
cctgcgagac	27	6	0	unique to 25	NO HITS[b]			
tcataaagca	26	6	0	unique to 25	NO HITS[b]			
tatatccatt	26	6	0	unique to 25	NO HITS[b]	2.00E-71		
tatcatccgt	26	6	0	unique to 25	Myo-inositol transporter A		9.00E-59	*N. crassa* AL390218
tatgatgttt	26	6	0	unique to 25	NO HITS[b]			
catctattcc	286	67	40	1.7	NO HITS[a]			
tatttgttgt	25	6	0	unique to 25	DNA-directed RNA polymerase II	1.00E-91		*S. pombe* D13337
ttagcgacag	25	6	0	unique to 25	NO HITS[b]			
gtttaatcaa	24	6	0	unique to 25	COPII-coated vesicle component	3.00E-18	7.00E-19	*S. pombe* AL109831
aatgactttt	427	100	68	1.5	NO HITS[b]			
tctttgatgt-5′	328	77	49	1.6	ADP, ATP carrier protein	1.00E-133	2.00E-72	*Gossypium hirsutum* AF006489
tgtcataaaa	23	5	0	unique to 25	NO HITS[b]			

3′ or 5′ denotes that a second serial analysis of gene expression (SAGE) tag was found and that the tag is either the 3′ most tag or more 5′. Tags in this table are differentially expressed with a statistic significance of $P < 0.05$.
[a]Tag did not have a corresponding contig at Stanford and did not have a corresponding expressed sequence tag (EST) at Oklahoma.
[b]No significant BLAST hit results for the genomic or EST sequence associated with the SAGE tag.

Tags With Higher Levels at 37°C

We also made preliminary gene assignments for 50 tags that showed statistically significant elevated levels at 37°C (Table 5). The tag with the greatest difference was approximately 47-fold higher at 37°C but represented a transcript from a putative open reading frame on sequence contig cneo010512.Contig5001 with no similarity to known genes. For the other tags, a number of categories of expression were noted that could reflect the adaptation of *C. neoformans* to growth at 37°C. This adaptation could include changes in the rate of protein synthesis because up-regulated tags matched transcripts for translation elongation factor-1α, a translation initiation factor, and three ribosomal proteins. As well, a change in protein synthesis correlated with the earlier observation that 12 and 11 ribosomal proteins were found in the 37°C libraries for both H99 and B3501, relative to three and four ribosomal proteins at 25°C for H99 and B3501, respectively.

We identified tags representing several heat shock proteins (HSP60, HSP70, HSP80) that had higher transcript levels at 37°C. This observation is particularly interesting in light of observations that heat shock proteins 60 and 70 have been identified as prominent antigens in animals and humans infected with *C. neoformans* (Kakeya et al. 1997, 1999). The expression of heat shock proteins appears to be a feature of growth in an animal host, and the in vitro growth conditions that we used for the SAGE libraries reflect the host conditions in this regard. The correlation between heat shock gene transcription and growth at 37°C is not absolute because we also observed one protein from the heat shock protein 12 family (HSP12) to be up-regulated at 25°C (Table 4). Interestingly, one of the highest BLASTP results for this putative *C. neoformans* Hsp12 showed 60% similarity with Wh11p from *C. albicans*; the expression of the gene for this protein is not regulated by temperature (Soll 1997). The influence of growth at 37°C on both translation elongation machinery and heat shock proteins is consistent with observations in *E. coli*. Farewell and Neidhardt (1998) have shown that the polypeptide elongation rate increases as a function of temperature and that the rate of elongation appears to be linked mechanistically to the heat shock response. An association between the expression of heat shock proteins and thermotolerance has also been noted in other fungal pathogens such as *H. capsulatum* (Caruso et al. 1987).

We also found that the collection of tags up-regulated at 37°C included genes for two proteases (carboxypeptidase D, serine protease) and a hydroxylase that may be involved in phenolic metabolism (putative salicylate hydroxylase). Our investigation of other tags not included in Table 5 also revealed that transcripts for enzymes involved in phenolic metabolism (aryl-alcohol dehydrogenase and cinnomoyl CoA reductase) were higher at 37°C (data not shown). These results indicate a relationship between growth temperature and the metabolism of phenolic compounds in *C. neoformans*. This may be related to the well-characterized ability of this fungus to convert diphenolic compounds into melanin (Salas et al. 1996; Casadevall and Perfect 1998).

Our results revealed that some genes predicted to encode proteins with iron as a cofactor (aconitase, ubiquinol-cytochrome C reductase) have higher transcript levels at 37°C (Table 5). In this regard, Perfect et al. (1998) found that the *C. neoformans COX1* gene encoding cytochrome C oxidase subunit 1 is up-regulated in a rabbit model of infection and during a temperature shift from 30°C to 37°C. This indicates an important role for mitochondrial function in the stress response of *C. neoformans*, and our observations indicate a general influence of temperature on respiration and iron homeostasis in *C. neoformans*. In further support of an influence on iron homeostasis, we observed a tag for a predicted iron permease that was elevated at 25°C. A similar theme regarding iron homeostasis has emerged from the global analysis of the influence of temperature on transcription in group A *Streptococcus* (Smoot et al. 2001). As indicated above, the parallels between the responses of group A *Streptococcus* and *C. neoformans* to elevated temperature also extended to the expression of the antioxidant protein superoxide dismutase. Our examination of the influence of temperature on gene expression in *C. neoformans*, although at a relatively early stage, indicates that striking parallels may exist with the response of group A *Streptococcus* to elevated temperature.

The 37°C B3501 library also contained a putative ortholog of a peripheral benzodiazepine receptor (2.68-fold higher at 37°C). The peripheral-type benzodiazepine receptor is localized to the outer mitochondrial membrane and is important for the regulation of cholesterol transport into the mitochondria, a rate-determining step in steroid biosynthesis (Li et al. 2001). Amino acid alignments showed conservation of the cholesterol-binding motif in the cytoplasmic C-terminal domain predicted from the *C. neoformans* sequence (data not shown). In this context, the elevated tag level for this gene might reflect an adaptation at 37°C that involves steroid metabolism; this observation is intriguing because of the elevated transcript levels that we observed at 25°C for genes involved in sterol biosynthesis.

Tags Representing Putative Regulatory Proteins

As indicated in Figures 1 and 2, many more tags than those analyzed so far are known to be present at different levels between the two temperatures. As part of our ongoing analysis of the SAGE tags for strain B3501, we performed an initial scan for tags that may represent genes for regulatory proteins in an additional 50 tags at each temperature. Although a complete analysis is not yet possible, we did match tags with genes for several putative proteins of interest. For example, we found a tag (elevated at 37°C) for a gene with similarity to an engrailed-related gene from insects (AATGGATTAA) that functions in development (Marie and Bacon 2000). We also found tags that were elevated at 37°C for two WD repeat proteins, one of which showed similarity to the Tup1p global repressor of *S. cerevisiae* (CAGACGCTGT) and the other to the Pop1p protein of *S. pombe* (Kominami et al. 1998). The possibility that a *TUP1*-like gene is regulated by temperature in *C. neoformans* is intriguing in light of the role of a *TUP1* ortholog in the filamentous growth of the fungal pathogen *C. albicans* (Braun and Johnson 1997). We should note, however, that a BLAST search of the *C. neoformans* genomic database with the Tup1p sequence of *C. albicans* revealed a gene with a greater level of sequence similarity than the one identified by our SAGE tag. The possibility of temperature control of a global regulator like Tup1p is interesting, however, because it has recently been shown that diploid strains of *C. neoformans* shows a temperature-dependent shift between budding (37°C) and filamentous growth (24°C; Sia et al. 2000). As we identify additional temperature regulated genes in our SAGE analysis, it will be possible to screen for *C. neoformans* or-

Table 5. B3501 Tags More Highly Expressed at 37°C

SAGE tag	B3501 37	B3501 25	25 normalized	FOLD difference	Preliminary gene designation	E-value Genome BLASTx	E-value EST BLASTx	Accession no. of BLASTx
atatgaaaga	55	5	1	55.0	NO HITS[b]			
aggaagagaa	125	186	44	2.8	Hypothetical protein		4.00E-22	*Agaris bisporus* AJ271701
acgtaccttt	41	21	5	8.2	NO HITS[b]			
cgacagaccg	207	529	124	1.7	Translation elongation factor 1α	0.00E + 00	0.00E + 00	*Cryptococcus neoformans* U81804
ggaatttgct	24	17	4	6.0	NO HITS[b]			
tagacagact	15	6	1	15.0	Carboxypeptidase D	1.00E-121		*P. janthinellum* AAB35195.1
accgacgtga	22	19	4	5.5	Aconitate hydratase	1.00E-165		*Piromyces sp* Y16747
cggaaaaaac	7	0	0	unique to 37	Hypothetical protein	1.00E-11		*Arabidopsis thaliana* AC002294
cctgttctcg	36	51	12	3.0	NO HITS[b]			
gccgcttctg	13	6	1	13.0	Ubiquinol-cytochrome C reductase iron-sulfur	1.00E-84		*Neurospora crassa* X02472
aaccagcggt	8	1	0	34.1	Salicylate hydroxylase	3.00E-12		*Streptomyces coelicolor* AL035707
aagacatcgt	9	2	0	19.2	NO HITS[b]			
attttagaaa	9	2	0	19.2	NADH-ubiquinone oxidoreductase subunit	6.00E-07		*N. crassa* X60829
gtccataagg	13	7	2	6.5	NO HITS[b]			
taactcgcat	6	0	0	unique to 37	NO HITS[b]			
tctaagtata	6	0	0	unique to 37	NO HITS[b]			
aacgtctgcc	45	80	19	2.4	NO HITS[a]			
cgcgcgatgc	16	14	3	5.3	NO HITS[b]			
gcattggcgt	39	70	16	2.4	ER chaperone BiP	0.00E + 00	5.00E-20	*Aspergillus oryzae* AB030231
catctggatg	5	0	0	unique to 37	NO HITS[b]			
tgttatcggt	16	15	4	4.0	Heat shock protein 80	1.00E-135		*N. crassa* AL513463
gcattttggg	18	20	5	3.6	Ubiquinol-cytochrome C	1.00E-34		*N. crassa* Y08841
aaccgcacca	29	46	11	2.6	Reductase core protein peripheral benzodiazepine receptor—human		6.00E-16	*Homo sapiens* JE0149
tgtagtatct	13	11	3	4.3	NO HITS[b]			
tcgagtttca	11	8	2	5.5	NO HITS[b]			
attgagatgg	44	91	21	2.1	NO HITS[b]			
ctaggttatg	4	0	0	unique to 37	3′ to 5′ DNA/RNA helicase	1.00E-165		*Schizosaccharomyces pombe* AL590902
ccgcctgccg	4	0	0	unique to 37	NO HITS[b]			
gctgcaagcg	4	0	0	unique to 37	Hypothetical protein	2.00E-43		*N. crassa* AL513463
ttcgcggtag	4	0	0	unique to 37	NO HITS[b]			
gtgatggtgg	4	0	0	unique to 37	NO HITS[b]			
ccctacgaga	4	0	0	unique to 37	NO HITS[a]			
atcgcgatgt	4	0	0	unique to 37	Putative protein	4.00E-05		*Mus musculus* NM_025872
gggagccata	4	0	0	unique to 37	NO HITS[b]			
atcctttgtc	4	0	0	unique to 37	NO HITS[b]			
actcaaccgt	10	7	2	5.0	NO HITS[b]			
catagttggt	27	47	11	2.5	Heat shock protein 70 family	0.00E + 00		*Malassezia sympodialis* AJ428052
ctcaagaagg	17	22	5	3.4	Subtilisin-like serine protease	4.00E-91		*Penicillium citrinum* AF098517
tcagaaccgt	6	2	0	12.8	NO HITS[b]			
cagaacaaag	5	1	0	21.3	protein with similarity to GAPDH	3.00E-28		*Mesorhizobium loti* AP003004
tatggctgga	5	1	0	21.3	NO HITS[b]			
gaagtccgga	5	1	0	21.3	NO HITS[b]			
tacactgtcg	12	12	3	4.0	NO HITS[b]			
gtttatggaa	11	11	3	3.7	Heat shock protein 60	0.00E + 00		*Coccidioides immitis* U81786
aacgtaaagc	7	4	1	7.0	NO HITS[b]			
gtgtggggca	7	4	1	7.0	NO HITS[b]			
caacgtagaa	7	4	1	7.0	NO HITS[b]			
tcatcaccat	7	4	1	7.0	Translation initiation factor 3	6.00E-15		*Myxococcus xanthus* AF261103
aactcgtgaa	15	20	5	3.0	Hypothetical protein	2.00E-22	5.00E-05	*A. bisporus* AJ271701
gcggtgggat	14	18	4	3.5	NO HITS[a]			

3′ or 5′ denotes that a second serial analysis of gene expression (SAGE) tag was found and that the tag is either the 3′ most tag or more 5′. Tags in this table are differentially expressed with a statistic significance of $P < 0.05$.
[a]Tag did not have a corresponding contig at Stanford and did not have a corresponding expressed sequence tag (EST) at Oklahoma.
[b]No significant BLAST hit results for the genomic or EST sequence associated with the SAGE tag.

tholog of genes known to regulated by Tup1p in *S. cerevisiae* and *C. albicans* (Braun et al. 2000; Wu et al. 2001).

Confirmation of SAGE Results by RNA Blot Analysis

RNA blot analysis was used to confirm that the observed differences in tag levels reflected differences in transcript levels. As shown in Figure 3A, the transcript level for a putative heat shock 70 protein was found to be elevated at 37°C compared with 25°C; this result was predicted by the SAGE data, which indicated an approximately twofold higher RNA expression at 37°C. Similarly, the RNA level detected for a predicted monosaccharide transporter gene from B3501 was found to be higher at 25°C compared with 37°C, as predicted by the SAGE results (~12-fold higher; Fig. 3B). The differential RNA levels indicated by the SAGE results were also confirmed by RNA blot analysis for eight additional genes, and all hybridization experiments were performed with two independent preparations of RNA from cells grown at the two temperatures (data not shown). Overall, the hybridization results support the conclusion that SAGE accurately identified genes with transcript levels that are influenced by temperature.

Summary

This report describes the first genome-wide analysis of the temperature-regulated transcriptome of *C. neoformans*. The results indicate that the transcript levels for a large number of genes are influenced by growth temperature in this fungal pathogen and that differences exist in the response of different varieties. Our data indicate that the fungus may respond to temperature with a change in chromatin packaging, as indicated by the differential transcript levels for histone genes. At 37°C, the fungus responds by elevating transcript levels for heat shock proteins, translation machinery components, mitochrondrial proteins, and stress proteins such as superoxide dismutase. These results indicate that elevated temperature is a stressful condition for this fungus. It will be interesting to examine whether this pattern is reinforced by a more detailed analysis of the H99 strain because isolates of this serotype (A) are more commonly associated with infections in North America, and strains of this serotype are generally more heat tolerant (Martinez et al. 2001). The completion and annotation of the genomic sequence for *C. neoformans* will allow a more detailed exploration of the generalities of the differential expression described above, and allow the identification of new patterns of temperature-regulated gene expression. Finally, even at this level of analysis at which the genomes of strains H99 and B3501 are only partially characterized, we noticed significant differences between the two strains and intriguing similarities with expression patterns for group A *Streptococcus* in terms of connections between temperature, iron homeostasis, and the stress response. These observations may reflect a general response of pathogens to growth at host temperature. Of course, the in vitro conditions used here do not adequately mimic the host environment, and transcriptional changes that reflect the pathogen response to the host immune system and host nutritional conditions may not be identified. To address this limitation, additional SAGE experiments are underway with *C. neoformans* cells isolated from infected animals or grown under iron limiting conditions. Finally, the SAGE tags generated in this study will be useful for the annotation of the Cryptococcus genome, particularly in the identification of transcribed regions.

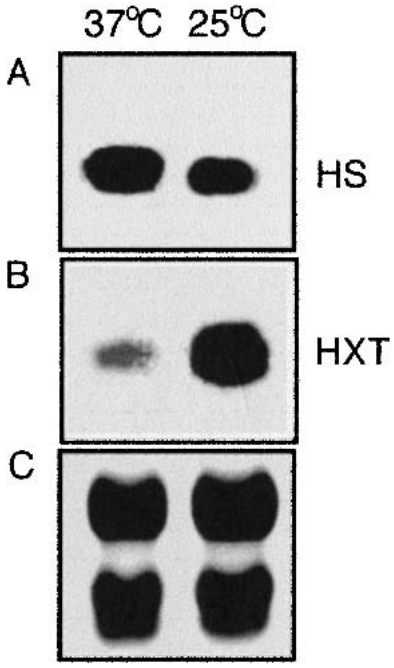

Figure 3 RNA blot analysis of two representative temperature-regulated genes in strain B3501. The RNA was isolated from cells grown at 25°C or 37°C. (*A*) Hybridization with a polymerase chain reaction (PCR) amplicon from a gene for a heat shock protein 70 (tag, CATAGTTGGT) with a higher transcript level at 37°C (HS). (*B*) Hybridization with a PCR amplicon from a gene for a high-affinity monosaccharide transporter (tag, GGCTTGACCA) with a higher transcript level at 25°C (HXT). (*C*) Ribosomal RNA (18S and 28S) bands as a loading control.

METHODS

Strains and Growth Conditions

C. neoformans serotype A, MATα strain H99 and serotype D, MATα strain B3501 were supplied by J. Heitman (Duke University) and J. Kwon-Chung (National Institutes of Health), respectively. For SAGE library construction, 2-mL cultures of yeast extract, peptone, dextrose broth were inoculated with single colonies and grown overnight at 30°C in a gyratory shaker (250 rpm). The cells from 1 mL of the culture were collected by centrifugation, washed twice with yeast nitrogen base broth, and resuspended in 1 mL of YNB buffered with 50 mM 3-[N-morpholino] propanesulfonic acid (pH 7.0). One hundred microliters of washed cells were used to inoculate 50 mL of the same medium in a sterilized 1-L Erlenmeyer flask. Cultures were grown at either 25°C or 37°C in a gyratory shaker until early log phase ($OD_{600} \cong 14.0$). The cells for mRNA isolation were in the exponential phase of growth, and the growth rate was similar at both temperatures (data not shown). Cells were harvested by centrifugation and immediately flash frozen in a dry ice–ethanol bath.

RNA Isolation and Analysis

Frozen cell pellets were lyophilized overnight at −20°C until dry and resuspended in 15 mL of TRIZOL extraction buffer (GIBCO BRL). Total RNA was isolated according to the manufacturer's recommendations with the addition of an overnight LiCl precipitation at 4°C following the standard ethanol precipitation step. PolyA+ RNA was isolated using the MessageMaker kit (GIBCO BRL). RNA blot preparation and hybridization was performed as described (Sambrook et al. 1989). A hybridization probe was prepared for a gene encod-

ing high-affinity monosaccharide transporter (tag, CATGGGCTTGACCA) using the primers 5′-AAGATAAGGAG TAATGACGGGCGA-3′ and 5′-CTATTGGTGAAATTTTCCCA-3′ (107-bp amplicon). The primers for the heat shock gene were 5′-ATGGTTCACCGACGTCCAGA-3′ and 5-′GCCACC GAAATGCCTGTCAT-3′ (262-bp amplicon). These DNAs were labeled with an Oligolabeling kit (Amersham Pharmacia Biotech Inc.).

SAGE Analysis

SAGE was performed as described by Velculescu et al. (1995) using the protocol available at www.sagenet.org. Poly-A RNA was converted to double-stranded cDNA using the GIBCO BRL synthesis kit and biotinylated oligo-dT_{18}. Briefly, the cDNA was cleaved with *Nla*III, the 3′-terminal cDNA fragments were bound to streptavidin beads (Dynal), and oligonucleotide linkers containing *Bsm*FI restriction sites were ligated to the 5′ ends. The linkered cDNA was released from the streptavidin bead by *Bsm*FI digestion, and tags were ligated to one another, polymerase chain reaction (PCR) amplified, concatemerized, and cloned into the *Sph*I site of pZERO 1.0 (Invitrogen). Twenty-eight PCR cycles were used to amplify ditags during library construction. Colonies were screened by PCR (M13F and M13R primers) to assess the average clone insert size and percentage of nonrecombinants. Tags were obtained by BigDye primer cycle sequencing and analysis on an ABI PRISM 3700 DNA analyzer. Sequence chromatograms were processed using `Phred` (Ewing and Green 1998; Ewing et al. 1998) and vector sequence detected using `CROSS_MATCH` (Gordon et al. 1998). Fourteen-bp tags were extracted from the vector clipped sequence, and an overall quality score for each tag was derived based on the cumulative `Phred` score. Duplicate di-tags and linker sequences were removed as decribed (Velculescu et al. 1995). Only tags with a predicted accuracy of ≥99% were used in this study. Statistical differences between tag abundance in different libraries was determined using the G-test (Sokal and Rohlf 1991) and the methods of Audic and Claverie (1997).

Tag Identification

To make preliminary assignments of tags to genes, we used the shotgun sequence data from the *C. neoformans* Genome Project (assemblies 010512 and 011005), Stanford Genome Technology Center (http://www-sequence.stanford.edu; funded by the National Institute of Allergy and Infectious Diseases (NIAID)/National Institutes of Health under cooperative agreement AI47087) and at TIGR (http://www.tigr.org/tdb/edb2/crypt/htmls/index.shtml). A limited amount of genomic shotgun sequence data is also available for strain H99 from our BAC clone end sequencing (see accompanying paper by Schein et al. in this issue) and at the Duke University Center for Genome Technology (http://cgt.genetics.duke.edu/data/index.html). In addition, limited EST databases are available for strains JEC21 and H99 at the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/cneo.html, funded under the cooperative agreement UO1 AI 485 94-01). We restricted our analysis to those genes for which an unambiguous tag assignment could be obtained either by annotation of the Stanford genomic data for JEC21 (assembly) or by analysis of ESTs from JEC21 or H99. `BLASTx` (basic local alignment search tool) results were recorded for those genes that had significant similarity with other proteins in the nonredundant database and National Center for Biotechnology Information (NCBI). Expect values and tentative gene assignments were recorded for those tags that were found to correspond to the 3′ most *Nla*III site within the putative open reading frame or within a 3′ untranslated region. In addition, the `BLASTx` results were inspected individually. In some cases, we found a high Expect value when the alignment of the protein from the nonredundant database and the *C. neoformans* sequence showed significant identity such that the Expect value did not reflect the extent of similarity. This occurred most frequently with small proteins. Because of the presence of introns in the genomic sequence and the length of the contigs, the Expect values recorded here are much lower than those that would be found if introns were removed, sequences were translated, and `BLASTp` analysis was performed. For the preliminary identification of ribosomal proteins, our nomenclature followed the outlined standards for *S. cerevisiae* (Mager et al. 1997). It should be noted that *C. neoformans* genes typically have an average of 5.6 introns per gene, and this complicates unambiguous identification of the 3′ end of genes. We did note that tags were often near a putative polyadenylation signal that corresponded with the consensus sequence AAC/GAAA similar to what has been observed previously (Chaturvedi et al. 2001).

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