

The complete cDNA sequence and expression of the first major allergenic protein of *Malassezia furfur*, Mal f 1

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For the first time the complete cDNA encoding a major allergen and novel protein of the yeast *Malassezia furfur*, Mal f 1, has been sequenced and expressed. The amino acid sequences of nine tryptic peptides of the protein were determined. Oligonucleotides were designed from these amino acid sequences. The cDNA sequence was obtained by hybridizing these primers to mRNA and enhancement by reverse-transcriptase PCR techniques. The cDNA is 1176 bp in length. It shows an open reading frame of 1050 bp coding for a protein of 38 178 Da and a deduced amino acid sequence containing 350 residues. The hydropathy plot and the tryptic digest indicate that the first 22 amino acids represent a leader sequence determining a mature protein of 35 988 Da. The complete encoding cDNA was expressed as a maltose-binding protein fusion protein in *Escherichia coli*. The recombinant fusion protein reacted with our specific monoclonal antibody and with IgE from patients with atopic dermatitis.

Keywords: allergy; yeast; *Malassezia furfur*; PCR; cDNA cloning; protein expression.

The yeast *Malassezia furfur*, previously also denoted *Pityrosporum orbiculare* or *Pityrosporum ovale*, is a natural inhabitant of the human skin microflora [1, 2]. However, under the influence of predisposing factors, this yeast may induce allergic reactions in atopic dermatitis. The importance of *M. furfur* in the pathogenesis of this disease has been investigated during the last 10 years and, in 40–65% of patients, activity against *M. furfur* has been shown with skin tests and/or specific IgE serology [3–8].

Numerous IgE-binding components with molecular masses between 10–100 kDa have been identified by immunochemical methods [9–12]. We have previously reported the production of a specific mouse monoclonal antibody (mAb 9G9) against one major allergen of *M. furfur* of approximately 37 kDa [9]. This protein is now designated as Mal f 1 according to the new allergen nomenclature [13]. This work reports the complete cDNA sequence of a novel protein from the yeast *M. furfur* that shows the criteria of a major allergen in atopic dermatitis.

MATERIALS AND METHODS

Isolation of the major allergen of *M. furfur*, Mal f 1. *M. furfur* from the American Type Culture Collection (no. 42132) was cultured on glycerol monostearate/olive oil medium for 2–

4 days at 37°C. The yeast extract was prepared as described earlier [9]. The proteins were reduced, alkylated with 50 mM iodoacetamide and separated on a 12.5% SDS/polyacrylamide gel. Proteins were visualized by Coomassie blue staining and the allergen verified by immunoblotting using our mAb 9G9.

Proteolytic digestion, fractionation of internal fragments.

The stained protein band was excised from the gel and subjected to in-gel digestion followed by separation of peptides [14]. Briefly, the gel piece was washed, dried and digested with 0.5 µg modified trypsin, sequence grade (Promega, Scandinavian Diagnostic Services) at 30°C overnight. The generated fragments were extracted and isolated by narrow-bore reverse-phase liquid chromatography using a column of µRPC C2/C18 SC 2.1/10 operated in a SMART system (Pharmacia Biotech). A linear gradient of acetonitrile (0.25%/min) in 0.06% trifluoroacetic acid was used at a flow rate of 100 µl/min to elute the peptides. The eluate was monitored at 215 nm and 280 nm.

ELISA assay. 54 fractions of the tryptic protein digest were analyzed by the standard ELISA technique using our specific mAb 9G9 [9]. Briefly, the peptides were coated onto an ELISA plate, blocked with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10.5 mM NaHPO₄, 1.7 mM KH₂PO₄, pH 7.4) containing 0.5% BSA and incubated overnight at room temperature with the mAb 9G9. After washing with 0.9% NaCl/0.5% Tween 20, the wells were incubated for 4 h at room temperature with alkaline-phosphatase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts). The substrate (1 mg/ml 4-nitrophenyl phosphate 0.1 M diethanolamine pH 9.8) was added and the absorbance at 405 nm was measured after 15 min in a microtiter plate reader (SLT-Lab Instruments).

Amino acid sequencing. The amino acid sequences of nine peptides, including the peptide recognized by the mAb 9G9, were determined by automated Edman degradation in an Applied Biosystems model 470A sequencer. The instrument was

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Abbreviations. 3' and 5'RACE, rapid amplification of cDNA 3' end and 5' end; MBP, maltose-binding protein; RAST, radio allergosorbent test.

Note. The novel nucleotide sequence data published here have been deposited with the EMBL sequence data bank and is available under accession number X96486.

operated following the manufacturer's instructions. The sequences are listed in Table 1.

Isolation of mRNA. The mRNA from *M. furfur* cultures was isolated using the PolyATtract system 1000 (Promega, Scandinavian Diagnostic Services). Briefly, approximately 0.3 g of a 2-days-old yeast culture [9] was homogenized in 4 ml extraction buffer. After the addition of 8 ml dilution buffer and 500 pmol biotinylated oligo(dT), the sample was incubated at 70°C for 5 min. The mixture was centrifuged at 12000×g for 10 min at room temperature, the supernatant was transferred to a clean tube (Falcon, 50 ml) and incubated with 6 ml streptavidin MagneSphere paramagnetic particles at room temperature for 2 min. Following three washes with 75 mM NaCl, 7.5 mM sodium citrate, pH 7.2, the magnetic particles were captured with a magnetic stand, and the mRNA eluted with 1 ml diethyl-pyrocabonate-treated water. The nucleic acid was then alcohol-precipitated, dissolved in water and stored at -70°C.

Construction of oligonucleotide primers. Two degenerate oligonucleotides (p1 and p2) were designed according to the amino acid sequences of peptide 1 and peptide 8 respectively. Both primers were in the sense orientation and used in a 3' rapid amplification of cDNA ends (RACE) protocol together with oligo(dT)1 and oligo(dT)2. For the 5' RACE protocol match primers, p3 and p4, and a 5' anchor primer (Gibco/BRL, Life Technologies) were utilized. Additional primers were designed, primers sp3-sp7 for internal sequencing and primers pe1 and pe2 for protein expression. All primers are listed in Table 2.

PCR amplification. Reverse-transcriptase PCR was used to amplify the RNA and to obtain three overlapping cDNA fragments using the following primer combinations: p1 and p2 together with oligo(dT)1 and oligo(dT)2; p3 and p4 with 5' RACE anchor primer.

The amplification of the two 3' fragments of Mal f 1 allergen cDNA was based on standard protocols. Briefly, mRNA was added to 1 µmol oligo(dT)1 in a 10-µl volume, heated to 70°C for 10 min and chilled on ice. The following solutions were added to the final concentration in first strand synthesis buffer (BRL): 0.5 mM of each dNTP and 10 mM dithiothreitol. After 2 min at 42°C, 200 U Superscript RNase H⁻ (BRL) were added and the reaction incubated for 30 min at 42°C. The mixture was heated to 55°C for 5 min, 2 U RNase H added, incubated 10 min at 55°C and then chilled on ice. The cDNA was purified using GlassMax columns (BRL). The PCR amplification was performed with 0.4 µM p1 or p2 and oligo(dT)2 in extender buffer (Stratagene, AH Diagnostic AB) containing 0.25 mM of each dNTP and overlaid with 1 vol. mineral oil. After an initial denaturation at 94°C for 8 min, 5 U *Taq* polymerase (BRL) and an equal amount of *Taq* extender (Stratagene) and Perfect match (Stratagene) was added at 80°C. The mixture was then subjected to 40 cycles of amplification in an MJ Research minicycler: 1 min at 94°C, 2 min at 55°C, and 2.5 min at 72°C.

The 5' fragments were obtained using p3 for the cDNA synthesis, then the fragments were tailed with 1 mM dCTP and 30 U terminal dideoxytransferase according to manufacturer's protocol (BRL). PCR amplifications were performed as described above using primer p4 and 5' anchor primer (BRL).

Cloning and sequencing of cDNA. The amplified products were analyzed by electrophoresis, and the positive samples were eluted from agarose gels. Isolated 3' and 5' fragment DNAs from PCR reactions were directly cloned into the TA3 plasmid using the TA cloning kit (Invitrogen NV). After transformation, plasmid DNA minipreparations from bacterial colonies were confirmed by restriction enzyme digestion.

Both strands of the recombinant plasmids were sequenced by the dideoxy chain-termination method using the Sequenase

2.0 kit (US Biochemical) with SP6, T7, and internal sequencing primers (listed in Table 2).

Expression of rMal f 1 in *Escherichia coli*. A full-length clone of Mal f 1 was obtained by reverse-transcriptase PCR using the downstream primer pe1 and the upstream primer pe2 (listed in Table 2). The conditions for the cDNA synthesis and for the PCR were the same as used for generating the overlapping fragments. The PCR product was ligated into the pMALc2 vector (New England Biolabs) into the *Bam*HI restriction enzyme site of the multiple cloning site. After transformation into competent XL2 Blue bacteria cells (Stratagene), the orientations of the inserts were analyzed by specific restriction enzyme digests with *Bam*HI and *Eco*RI. The nucleotide sequence of the full-length clone was confirmed by DNA sequencing.

A rMal f 1 bacterial culture was grown overnight in Luria-Bertani medium supplemented with ampicillin (100 µg/ml) at 37°C, then diluted 50 times and continued to grow under the same condition to a cell density of $A_{600} = 0.5$. The protein production was induced with 0.1 mM (final concentration) isopropyl β -D-thiogalactoside and the incubation continued for 2 h at 37°C.

Purification of rMal f 1 and immunoblotting. The purification of the fusion protein was based on the maltose-binding protein (MBP) at the N-terminus of the fusion protein, using the MBP fusion and purification system (New England Biolabs) following the manufacturer's protocol. Briefly, 50 ml cell culture, induced for fusion protein production as described above, was harvested by centrifugation at 4000×g for 10 min. The cell pellet was resuspended in 2.5 ml column buffer (20 mM Tris/HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen overnight at -20°C. The cells were thawed in a cold water bath and then subjected to sonication until the cells were completely lysed. Cell debris was removed from the sonicate by centrifugation at 9000×g for 30 min at 4°C; 50 µl of the clarified supernatant was transferred to a fresh tube and mixed with an equal volume of amylose resin. Following a 15-min incubation on ice, the sample was spun briefly. The supernatant was discarded and the resin washed in two 1-ml portions of column buffer. The resin was resuspended in 50 µl SDS/PAGE sample buffer and boiled for 5 min.

The purified proteins were separated on a 10% SDS/polyacrylamide gel under reducing conditions and transferred to Immobilon P membrane (Millipore AB). The immuno staining was performed as described earlier [9].

Patient sera and myeloma IgE. Sera from 15 atopic dermatitis patients with positive radio allergosorbent test (RAST) values (1.4–24.5 kU/l, RAST classes 2–4) to *M. furfur* were pooled. A pool from four patient sera with positive IgE levels to different allergens but negative to *M. furfur* was used as a negative control. The sera were diluted sixfold for the western blot experiments.

Myeloma IgE was a kind gift from Dr Carl Magnusson (Division of Clinical Immunology, Karolinska Hospital). It was diluted in cord serum to a concentration of 1000 kU/l and used as an additional negative control.

RESULTS AND DISCUSSION

To obtain the complete cDNA sequence of Mal f 1 major allergen, we utilized reverse transcriptase PCR, standard cloning and sequencing techniques. We isolated the protein from SDS/polyacrylamide gels, subjected it to tryptic digestion and fractionated the different peptides. We determined the epitope of our specific mAb 9G9 using the peptides and standard ELISA techniques. One fraction, listed as peptide 9 in Table 1, showed

Table 1. List of amino acid sequences obtained from trypsin-digested protein.

Peptide	Sequence of peptide
Peptide 1	XWDNWK
Peptide 2	LIAFGGPR
Peptide 3	ALTAFDVSKPYA
Peptide 4	APYAISFR
Peptide 5	IVTVPVGNESVLVGA
Peptide 6	SDYPLYK
Peptide 7	NLTPEDTIYDR
Peptide 8	ALPDQIDVK
Peptide 9	XFNFADQSS

Table 2. List of primers used in PCR.

Primer	Sequence
p1	5'-TGGGA (T/C) AA (T/C) TGGAA-3'
p2	5'-CCNGA (T/C) CA (A/G) AT (T/C/A) GA (T/C) GTNAA-3'
p3	5'-TTAAAAGTTTCAGGATGCTGTTGTCA-3'
p4	5'-CAGTAAAGCCACTGTTCT-3'
Oligo(dT)1	5'-TCTGAATTCTCGAGTCGACATCTTTTTTTTTT-3'
Oligo(dT)2	5'-TCTGAATTCTCGAGTCGACATCT-3'
Anchor primer (BRL):	5'-CUACUACUACUAGGCCACGCGTCGACTAGTAC-GGGIIGGGIIGGGIIG-3'
sp 3	5'-CAGGAGAGACAGTCCCGACA-3'
sp 4	5'-GCCTACCGGGACCGTAACAA-3'
sp 5	5'-ATGTCGGGACTGTCTCTCTCT-3'
sp 6	5'-GTTTGAAAAGAAGGCTGGCA-3'
sp 7	5'-AGGCACGCTTTCGGGTA-3'
pe 1	5'-GAGAGATCTTTAAAAGTTTCAGGATGCTGTTGT-CAAGCT
pe 2	5'-GAGGGATCCATGCGATACAGCACCGTCTTGGCA

a strong absorbance at 405 nm of 2.8 with mAb 9G9. We determined the amino acid sequence of nine peptides including the peptide reacting with mAb 9G9. All peptide sequences are listed in Table 1. We then amplified two different 3' cDNA fragments using degenerate primers p1 and p2 that were designed according to peptide 1 and peptide 8. These two fragments were aligned and the primers for the 5' RACE were designed according to the resulting sequence. The amplifications of the 5' fragments were performed using primer p3 for cDNA synthesis followed by the nested primer p4 in combination with the anchor primer (BRL). At least two independent PCR reactions were performed for the 3' RACE and the 5' RACE.

The cloned PCR fragments were sequenced and analyzed using the DNASIS (National Biosciences) and Genetic Computer Group (Wisconsin) software packages. The independent clones of each fragment showed the same sequence and the resulting fragments were linked to obtain the entire cDNA sequence. The full cDNA is 1176 nucleotides in length (Fig. 1) including 102 nucleotides after the stop codon up to the beginning of the poly(A) tail. The bracketed nucleotides immediately preceding the poly(A) tail (Fig. 1) indicate the nucleotide sequence found in the longest clone. A second independent clone was missing the bracketed nucleotides. These differences may be due to variability in the site of the oligo(dT) primer annealing. In higher eukaryotes the canonical polyadenylation signal AATAAA is predominant; however, in yeast, a unique process-

1	M R Y S T V L A A	9
1	AGCCTTTTACGACGAACCTAGCATGCGATACAGCACCGCTTGGCAGCG	48
10	L A L L G T S A V S V L A A L P	25
49	CTGGCTCTGCTCGGCACTTCGGCAGTTAGTGCTTGGCAGCTCTCCCT	96
26	D Q I D V K V K N L T P E D T I	41
97	GATCAGATTGATGTCAAGGTCAAGAATTGACCGGAGGACACAAT	144
42	Y D R T R Q V F Y Q S N L Y K G	57
145	TATGACCGTACTCGCAGGTGTTCTACCAATCGAATCTGTACAAAGGC	192
58	R I E V Y N P K T Q S H F N V V	73
193	CGCATTGAGGTCTACAACCCAAAGACCAGTCACACTTATGTGGTC	240
74	I D G A S S N G D G E Q Q M S G	89
241	ATTGACGGTGCTCGTCCATGGCGATGGTGAGCAGCAGATGTCGGGA	288
90	L S L L T H D N S K R L F A V M	105
289	CTGTCTCTCCGTACCCACGACAACCTGAAGCGTTTGTTCGCTGTTATG	336
106	K N A K S F N F A D Q S S H G A	121
337	AAGAATGCGAAGTCGTCAACTTTGCCGACCAGTCGAGCCATGTTGCA	384
122	S S F H S F N L P L S E N S K P	137
385	AGCTCGTTCACAGCTTCAACCTCCCTCTCTCGGAGAACAGCAAGCCT	432
138	V W S V N F E K V Q D E F E K K	153
433	GTCTGGTCTGTAACCTTCGAGAAGTCCAGGACGAGTTTGAAGAAG	480
154	A G K R P F G V V Q S A Q D R D	169
481	GCTGGCAAGCGCCCTTCGGTGTGGTGCAGTCGGCTCAGGACCGTGAT	528
170	G N S Y V A F A L G M P A I A R	185
529	GGCAACTCTTACGTGGCCTTTGCACTGGGTATGCCGCCATTGCCCGT	576
186	V S A D G K T V S T F A W E S G	201
577	GTGAGTGCTGATGGAAGACGGTGAGCACCTTTGCTGGGAATCGGGC	624
202	N G G Q R P G Y S G I T F D P H	217
625	AATGGAGGTGAGCGTCCCGGTACTCAGGTATCAGTTCGACCCCTCAC	672
218	S N K L I A F G G T G P R A L T A F	233
673	TCGAACAAGCTGATCGCGTTTGGTGGCCCTCGCGCCCTGACTGCCTTC	720
234	D V S K P Y A W P E P V K I N G	249
721	GATGTGAGCAAGCCTTATGCATGGCGGAGCCTGTTAAGATTACGGC	768
250	D F G T L S G T E K I V T V P V	265
769	GACTTTGGCAGCTTTCGGGTACCGAGAAGATTGTACGGTCCCGGTA	816
266	G N E S V L V G A R A P Y A I S	281
817	GGCAATGAGAGTGCTGGTGGGTGCTCGCGCCCTACGCCATTTCG	864
282	F R S W D N W K S A N I K K T K	297
865	TTCCGTTCTGGGACAACCTGGAAGTCGGCAACATCAAGAAGACCAAG	912
298	R S E L Q N S G F T A V A D Y Y	313
913	CGTTCGGAGTCGACAACAGTGGCTTTACTGCCGTGCCGACTACTAC	960
314	Q G S E Q G L Y A V S A F F D N	329
961	CAAGGCTCCGAGCAAGGTCTCTACGCTGTGAGCGCCTCTTCGACAAC	1008
330	G A H G G R S D Y P L Y K L D N	345
1009	GGCGCGCATGGTGGCGCAGCGACTACCCCTTGTACAAGCTTGACAAC	1056
346	S I L N F *	351
1057	AGCATCTCTGAACCTTTAAGTCAGAGAGCGATCAATTCTCTAGGCAATA	1104
1105	CGAAGGTGAACCATAGTCTGTCTGGGTAGTCGACTCAGCTGTTGTTTA	1152
1153	TCC[AAATACATGTTGATAACTTGCAA]AAAAAAA	1186

Fig. 1. cDNA sequence and translated amino acid sequence for the yeast allergen Mal f 1. The asterisk indicates the stop codon. The 21 nucleotides in brackets before the poly(A) tail represent the deletion area found in different clones.

ing and polyadenylation signal is absent [15–17]. The 5' non-translated sequence is 21 nucleotides in length and overcomes the critical leader length in yeast for ribosomes to initiate at the first AUG met regardless of the context [18]. The inferred amino acid sequence contains all nine determined peptide sequences and the calculated molecular mass of the protein is 38 177 Da.

The cDNA sequence was compared with other nucleotide sequences using the NCBI Blast E-mail search and GCG BLASTN [19]. No significant similarity was found with reported sequences. In addition, no other nucleic acid or protein sequences from the yeast *M. furfur* could be found in the com-

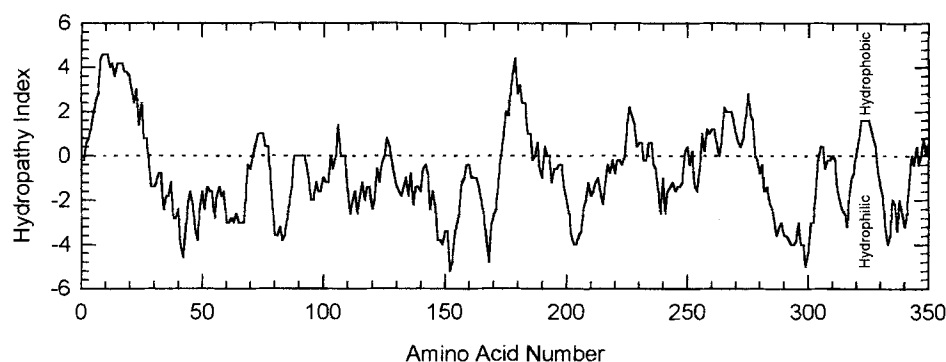


Fig. 2. Hydropathy profile of Mal f 1 deduced from the amino acid sequence.

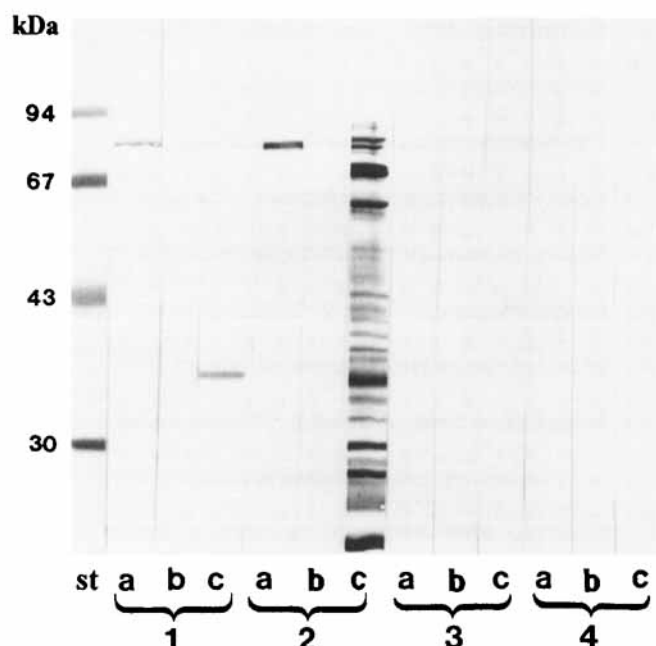


Fig. 3. Immunoblots with mAb 9G9 and IgE antibodies. (st) Molecular mass standard; (a) MBP-Mal f 1 fusion protein as antigen; (b) MBP as antigen; (c) total protein extract from *M. furfur* as antigen; (1) mAb 9G9; (2) *M. furfur* positive IgE serum pool; (3) *M. furfur* negative IgE serum pool; (4) myeloma IgE.

mon data bases so this may be the first gene sequence reported from this organism.

Mal f 1 is a major allergen of the yeast *M. furfur* [9]. We have reported that the highest expression of the protein is in the exponential phase of the yeast culture [20] and recently determined the localization of this protein in the cell wall and that it is exposed on the cell surface using confocal microscopy and flow cytometry [21]. A hydropathy plot of Mal f 1 based on algorithms' development by Kyte and Doolittle [22] using AN-THEPROT software with a window setting 5 is shown in Fig. 2. It reveals a hydrophobic region of sufficient length at the N-terminus to be a candidate for a membrane protein or a secreted cell wall protein. The tryptic peptide 8 is preceded by the amino acid alanine which additionally suggests that the protein is secreted, and the first 22 amino acids function as a leader sequence. If the first 22 amino acids represent a leader peptide, the mature protein has a molecular mass of 35988 Da. Further analysis of this protein shows one potential N-glycosylation site at amino acids 267–270 (Fig. 1). However, it has been shown recently that if the X in the classical site N-X-S/T is represented

by Trp, Asp, Glu or Leu, the glycosylation is inefficient [23]. A second site following the consensus sequence N-X-S/T can be observed at amino acid position 34–36; however, this sequence site is followed by Pro, and it has been shown that about 50% of the sites that have Pro C-terminal to the S/T are not glycosylated [24]. *In vitro* translation experiments with rabbit reticulocyte lysate in the presence of dog pancreatic microsomes suggest that the Mal f1 allergen is indeed very inefficiently glycosylated (unpublished data). The discrepancy between the calculated molecular mass of 36 kDa and the previously reported apparent molecular mass of 37 kDa is probably therefore due to inaccuracies inherent to molecular mass determination by SDS/PAGE.

In order to prove that the cloned cDNA sequence is indeed coding for an allergen, we have produced the full-length clone, including the probable leader peptide. We expressed the protein as a MBP fusion protein in *E. coli* and subjected it to immunoblotting experiments (Fig. 3). The lanes represent the MBP–Mal-f1 fusion protein (labeled a), MBP protein alone (labeled b) and total *M. furfur* protein extract (labeled c). Group 1 demonstrates a positive reaction with our monoclonal antibody with the recombinant fusion protein and the allergen in the natural extract. Group 2 shows a positive staining with the *M. furfur* reactive IgE serum pool of the fusion protein and the variety of allergens found in the *M. furfur* extract. The recombinant fusion protein shows a strong reaction with both the mAb 9G9 and with the IgE serum pool. No staining can be observed with MBP alone with either the mAb or the atopic dermatitis patient sera. The negative control group 3 (*M. furfur* negative sera) and group 4 (myeloma IgE) reacted with none of the applied proteins.

Here, we report the successful cloning, sequencing and expression of the major allergen Mal f 1 from the yeast *M. furfur*. With similar methods it should be possible to sequence the cDNAs coding for other allergens of this organism [9]. Our laboratory has raised several T-cell clones from a patient with atopic dermatitis specifically reacting with *M. furfur* [25]. These clones will be employed in T-cell epitope studies of this allergen. Knowledge of the sequence and the ability to express fragments of the molecule that bypass the B-cell response may allow the production of relatively non-allergic peptides that can be used to down-regulate the production of IgE antibodies [26]. The recombinant proteins could be used for diagnosis and lead to therapeutic tools as a mixture of T-cell epitope peptides.

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