

Northern blot identification of mRNA containing sequence for protein allergen, Alt a1, in eight strains of *Alternaria alternata*

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Background: Concentrations of Alt a1, a major allergen from *Alternaria alternata* extracts, exhibit significant batch-to-batch variability. This variability may result from basic strain-related genetic differences or from environmental influences such as growth conditions or extraction methods.

Objective: The objective of this work is to determine if strain-to-strain allergen variability in *Alternaria alternata*, Alt a1 allergen occurs at the nucleic acid level.

Methods: We compared the content of mRNA for a segment known to contain sequence coding for the reported N-termini of this allergen in eight strains of *Alternaria* obtained from three individual sources grown under identical conditions. RNA was extracted from rapidly growing mycelia and analyzed by northern blot analysis for the content of a specific segment containing sequence for the Alt a1 allergen. Blots were also analyzed for the content of a specific 5.8S rRNA segment.

Results: The size of the mRNA for this protein was about 700 bases. Sequence for the specific segment was found in all of the eight strains tested. When normalized for extraction variabilities using the content of 5.8S rRNA, the calculated concentrations of Alt a1 mRNA of seven of the eight strains were similar.

Conclusions: The results of these experiments suggest that this Alt a1 related allergen sequence is found in many strains of *Alternaria* and that variabilities in allergen protein content noted previously result from posttranslational events.

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INTRODUCTION

Alternaria alternata, a member of the fungi imperfecti, is a known cause of allergic symptoms in sensitive humans.¹ When grown in culture, numerous studies have demonstrated substantial interstrain differences in protein and allergen content as well as allergen content differences caused by extraction procedures.²⁻¹² There are many possible reasons for these differences including the changeable nature of the organism when grown under various conditions and the action of proteolytic and glycolytic enzymes, which increase the apparent complexity of the mixture.¹³

The yield of its major allergen, Alt a1, has been reported to be unaffected by use of vigorous physical measures to break up the mold, the presence of protease inhibitors, and phenol binding agents.¹⁰ These same authors were able to increase the yield of this specific allergen by careful selection of strain and growth conditions. Given the above result, we hypothesize that genetic differences that affect either the transcription of mRNA or translation of protein are present in different strains. These differences have a strong impact on the production of Alt a1. To test this hypothesis, we collected eight strains of *Alternaria* from different investigators and measured the amount of mRNA coding for Alt a1.

METHODS

Alternaria Source Materials

Eight strains of *Alternaria* were obtained from three different research

groups in North America. Three of the strains were available from ATCC, one was available from CBS (Netherlands) and four of the strains were derived from the wild or from other laboratory cultures. The designations of these are shown in Table 1.

Growth and Extraction

Cultures were grown in a low nutrient synthetically defined media containing only inorganic nitrogen, sucrose, and trace minerals¹⁴ for 1 week before harvesting. Air was continuously bubbled through the media and cultures were stirred constantly as described by Paris et al.¹⁰ Rapidly expanding mycelia were then separated from the culture medium by filtration through a Whatman 4 filter. The resultant dark brown mycelial mats were frozen at -70 °C in 2.5 to 2.75-gram aliquots. Mycelia were ground in liquid nitrogen, denatured in guanidine thiocyanate in the presence of RNase inhibitors, and disrupted with short bursts in a high speed homogenizer. Ribonucleic acid nucleic acid contamination was avoided by use of diethyl pyrocarbonate. Ribonucleic acid was extracted using a Total RNA Isolation Kit (Promega Corporation, Madison, WI) according to the instructions supplied by the manufacturer. Ribonucleic acid was pelleted by centrifugation, washed in 75% ice-cold ethanol, re-centrifuged, dried in a vacuum desiccator, and resuspended in 1 mL of RNase-free water. The purified RNA was stored at -70 °C.

Northern Blotting

Concentrations of RNA preparations were estimated by absorbency at 260 nm and equal amounts of total RNA (40 µg per lane) were applied to a formaldehyde denaturing agarose gel.

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Electrophoresed DNA was blotted by capillary action onto Nylon 66 membranes and stabilized by UV crosslinking. Labeled probe material produced was allowed to hybridize with the blotted RNA overnight at 65 °C. After washing, blots were visualized on a Molecular Dynamics phosphorimager and analyzed with the Image Quant program (Molecular Dynamics, Sunnyvale, CA, USA).

Probe Construction

Ribonucleic acid isolated from *Alternaria*, strain AL005, was used to produce cDNA fragments for the construction of probes. These fragments were created by reverse transcription using random hexamers (Pharmacia). Using PCR primers constructed by the Core facility at the University of Missouri at Kansas City, a 248-BP DNA fragment (ALT probe) (Fig 1) which contains the reported sequence for an N-terminal portion of Alt a1^{15,16} and a 156 BP DNA fragment from 5.8S rRNA¹⁷ (Fig 2) were amplified by PCR (Table 2). The sequence of the ALT probe is contained in the sequence of the *Alternaria alternata* allergenic protein Gene Bank accession number U67097 (Fig 1) sequenced from a 507-base pair insert derived from a c-DNA library constructed from *Alternaria* strain CBS-10333. These products were purified by electrophoresis and concentrated using a Wizard kit (Promega). The resultant DNA to be used for probing was radiolabeled using a DecaPrime Kit from Ambion. Ribonucleic acid blots were probed with the radiolabeled 248-BP DNA fragment, stripped, and then reprobed with the 156 BP DNA fragment. Regions of probe binding were identified by phosphorimager, densitometrically integrated, and the relative content of the Alt probe was compared with that of the 5.8S rRNA probe. Probe sequence as well as sequence for the 507-base pair insert derived from m-RNA of *Alternaria alternata* strain CBS-10333 was determined by the sequencing facility of the University of Missouri at Kansas City using an ABI 377 Auto-

Table 1. Source of Eight *Alternaria* Strains Studied

Strain	Source	Investigator
188698	Wild	H. Vijay*
188699	Wild	H. Vijay
188700	Wild	H. Vijay
188701	Wild	H. Vijay
ATCC 11680	ATCC 11680	R. Escht†
CBS 103-33	CBS 103-33 (Netherlands)	R. Esch
AL005	ATCC 96343	J. Portnoy
AL003	ATCC 96345	J. Portnoy‡

* Drug Toxicology Division. Bureau of Drug Research. Ottawa, Ontario, Canada.

† Greer Labs, Lenoir, North Carolina.

‡ Children's Mercy Hospital. Kansas City, Missouri.

	10	20	30	40	50	60
Alt U67097	CCAACGCTTC	CCGAAGTTCT	ATGCATCAGC	TACGAACGAT	ACGGCCCCGTG	ACCACCCAGG
Alt Probe					<u>CCCGTG</u>	<u>ACCACCCAGG</u>
	70	80	90	100	110	120
Alt U67097	GCGACTACGT	GTCCAAGATC	GNNACTAGCC	AGAGCTGGAG	GGAAGAGGAC	GGAGGCCATG
Alt Probe	<u>GCGACTACGT</u>	<u>GTCCAAGATC</u>	---A---GCC	AGAGCT--AC	GGAAGAAAGC	CGAGGCCATG
	130	140	150	160	170	180
Alt U67097	CTCAAGGAAG	AAGGGTGCCA	ACTAAAGTGG	TCGTGATATA	TAACGATTTC	GATACCCCGC
Alt Probe	CTCAA-GAAG	AAGGGTGCCA	ACTAAAGTGG	TCGTGATATA	TAACGATTTC	GATACCCCGC
	190	200	210	220	230	240
Alt U67097	ACACAGCACA	RTGTGCTAGG	AGTTGGAGGA	GGAAGATAAA	TAACTTGGAT	TTGACGATGA
Alt Probe	ACACAGCACA	GTGTGCTAGG	AGTTGGAGGA	GGAAGATAAA	TGACTTGGAT	TTGACGATGA
	250	260	270	280	290	300
Alt U67097	AACGTCCTCA	-----	TGAAGCCGGC	CTGAGACACG	GCAAAGGCAC	RAATGATGAA
Alt Probe	AACGTCCTCA	ATGAAC	TGAAGCCGGC	CTGAGACACG	<u>GCAAAGGCAC</u>	<u>RAATGATGAA</u>
	310	320	330	340	350	360
Alt U67097	GATCCGTACG	GATCCAACCT	TTAGCAGCCG	CAATCTGCGC	CGTATCTCCA	ATGGTGTTCAT
	370	380	390	400	410	420
Alt U67097	CGAAATTGAC	GGCCCCAGGAA	CACAAGATCC	AAGGGATCGG	GTACYGGTGC	GCTGTCTGTCG
	430	440	450	460	470	480
Alt U67097	CTGGCTGATG	CACTGAGGAA	GTCTGGGCAT	CGACCTTGAC	GTTGTGGACT	ACAACGAGCA
	490	500				
Alt U67097	CWCCAACGGT	ACAAGCAAAG	ACGCAAA			

Figure 1. Alignment of the *Alternaria* sequence U67097 containing a region previously identified as the N-terminal of the Alt a1 allergen with the sequence of the PCR derived probe used in the Northern blotting studies. The PCR primers used to synthesize the DNA needed for probe production are underlined. The probe represents 248 bases starting at position 44 of the complete sequence and ending at position 295. There are two areas of nonalignment in the sequence, two 3 base deletions at position 18-87 and a 7 base deletion at position 250.

mated DNA sequencer and dye termination reaction system.

Allergen Enzyme Immuno-Assay

Two-gram aliquots of mycelia that had been stored at -70 °C were thawed, dried at room temperature for two days, ground with mortar and pestle, and extracted for three hours at room temperature in 20 mL 0.1% ammonium bicarbonate. The insoluble material was removed by filtration through

a succession of decreasing pore size filters (Gelman, Sciences, Ann Arbor, MI, USA) ending with a 0.2-micron filter. The extract was then freeze dried and reconstituted at 10 mg dry weight per milliliter in phosphate buffered saline at pH 7.4. Specific *Alternaria* allergen, Alt a1, was measured using a double monoclonal antibody-based sandwich assay as described previously.¹⁸ Briefly, microtiter plates were

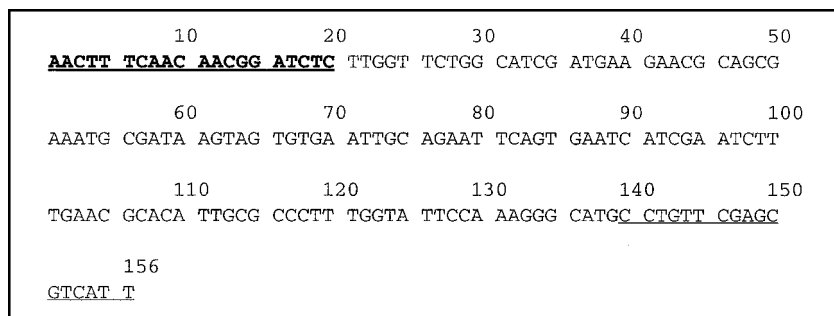


Figure 2. The sequence of the 156 BP DNA fragment used to probe for 5.8 S rRNA. The PCR primers used to synthesize the DNA needed for probe production are underlined.

Table 2. PCR* Primers and Conditions

PCR Methods

Oligonucleotides used for PCR included

1. The 31kD N-terminal oligo
5'-CCC GTG ACC ACC CAG GGC GAC TAC GTG TCC A-3'
2. The second strand reverse oligo for the Alt probe.
5'-CGG AAT TCA TCA TTT GTG CCT TTG CC-3'
3. The 5.8 S rRNA forward oligo.
5'-AAC TTT CAA CAA CGG ATC TC-3'
4. The 5.8 S rRNA reverse oligo.
5'-AAT GAC GCT CGA ACA GG-3'

* PCR program was one minute at 94 °C, and 30 cycles of 40 seconds at 92 °C, one minute at 60 °C, and one and one-half minutes at 75 °C for the Alt probe and 30 cycles of 30 seconds at 94 °C, 30 seconds at 65 °C, and one minute at 72 °C for the 5.8 S probe.

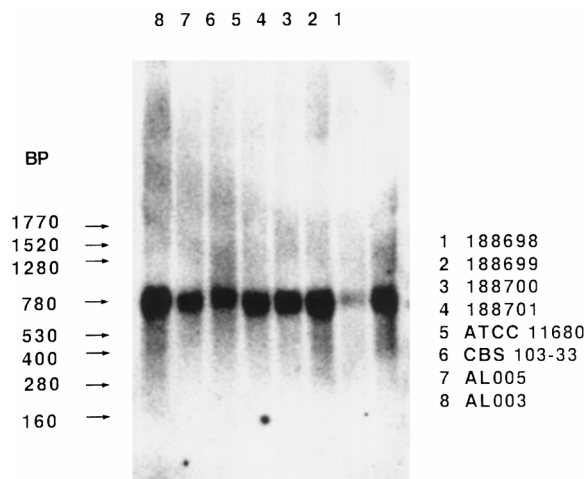


Figure 3. The computer-scanned image of the Northern blot probed with the 248-bp probe containing the Alt a1 sequence. The image is reproduced directly from the phosphorimager. The lanes are numbered right to left with the numbers representing the strain designations listed at right. The size markers at left are aligned with marks made on the blot after staining with ethidium bromide.

coated with the F(ab)2'-fragment of Mab1 overnight. Coated plates were washed and blocked with 0.5% gelatin (BioRAD) for one hour at 37 °C to reduce background. Serial dilutions of

ALT or purified allergen protein⁹ dissolved in PTA were added to each well and incubated for two hours. After washing, the plates were incubated with biotinylated Fab fragment of

Mab2 for two hours. The plates were again washed and incubated for one hour with 1:1000 avidin conjugated alkaline phosphatase (Sigma). The enzyme activity was detected at 405 nm on a Dynateck MR7000 reader. Standard curves were established and unknown concentrations were calculated using the DIAS software (Dynateck, Chantilly, VA). The assay is sensitive to Alt a1 concentrations as low as 0.2 µg/mL and is very specific for the allergen. The allergen detected by this assay is shown to bind to IgE from patients sensitive to *Alternaria* and is skin test positive in 15 of 16 patients also skin test positive to commercial *Alternaria* extracts.

RESULTS

The method of grinding the mycelia in liquid nitrogen and immediately homogenizing in the presence of RNAase denaturing agents produced adequate amounts of RNA from each strain. Some adjustments were made in the size of the sample application to accommodate for differences in the yield of total RNA, but these were slight. The sequence for the 248 bp PCR-derived probe produced from the AL005 strain is contained in the sequence derived from the CBS-10333 strain Gene Bank Accession #U67097. This probe, which contains the reported N-terminal sequence of the allergen, Alt a1 (Fig 1), binds to the same size mRNA in each of the eight strains (Fig 3). Blots containing purified RNA from *Alternaria* where RNA from *Epicoccum* and *Pithomyces* was used as negative control (data not shown) had probe binding only in those lanes containing *Alternaria*. The size of the Alt a1 mRNA appears to be about 700 BP (±10%). When integrated densitometrically, blots of Alt a1 mRNA in eight strains varied as much as 4-fold (Fig 5). This was due mostly to a sharply reduced amount in strain 188699. The rRNA segment was also found in all strains (Fig 4) and did not vary significantly between strains (Fig 5). When Alt a1 mRNA was normalized for overall concentration effects by dividing these amounts by the

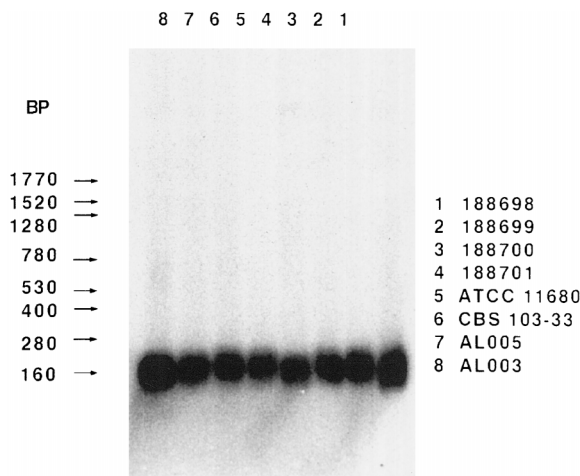


Figure 4. The computer scanned image of the Northern blot probed with the 156 bp probe for 5.8 S rRNA. Again, the image is reproduced directly from the phosphorimager. The lanes are numbered right to left and the numbers represent the strain designations represented at right. The size markers at left have been superimposed on the blot and aligned with known size markers.

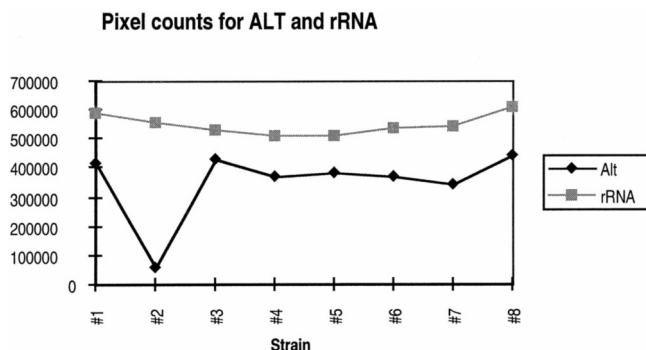


Figure 5. Graph of mRNA and Alt a1 of eight ALT strains (cellular antigen). The blots were integrated using the Image Quant software available with the phosphorimager. The pixel count number for each band on the two gels is represented on the y axis and the strain designation is represented on the x axis. The strain designation number is the same as in figures 4 and 5.

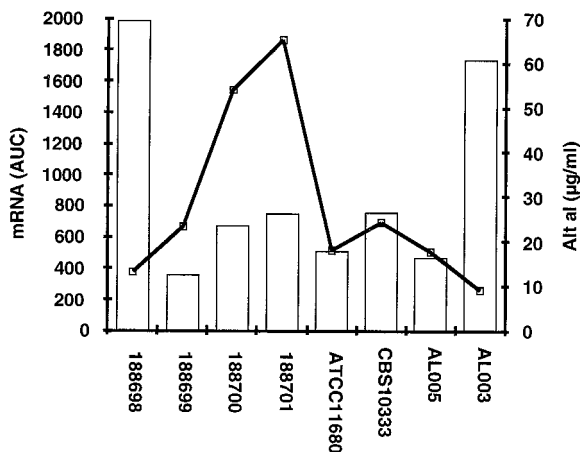


Figure 6. Graph of mRNA (bars) and Alt a1 (line) of eight Alt strains (cellular antigen). The blots were integrated using the Image Quant software available with the phosphorimager. The pixel count number or protein content is represented on the y axis and the strain designation is represented on the x axis.

corresponding amounts of rRNA in each strain, the lowered amount in strain 188699 became more pronounced. The amount of Alt a1 measured in the mycelia of these strains determined by EIA corresponded to the amount of mRNA coding for this allergen (Fig 6). There were notable discrepancies as with 188698 and AL003, which have the highest measured amounts of mRNA and the lowest measured amounts of allergen protein.

DISCUSSION

The most significant finding was that the same mRNA coding for the sequence represented in the probe was detected as a single band in all of the eight strains of *Alternaria*. The size of the mRNA varied slightly but averaged about 700 base pairs. This was slightly longer than the 507 bases in the reported sequence.

It is also significant that previous results¹⁷ concerning the sequence of rRNA for this fungus and the ubiquity of this sequence were confirmed. The probe containing DNA sequence coding for a portion of the rRNA for this fungus bound to the RNA blot at about 156 base pairs as expected. This rRNA was produced strongly by all mycelial preparations and had a sequence that did not vary significantly from strain to strain. As expected the results were consistent with previously published accounts.

The amount of mRNA for this allergen produced in the eight strains studied here varied significantly; but, the variation was seen mostly in the lowered amount of only one strain. The other seven strains showed remarkably similar levels of allergen mRNA especially when the total concentration differences were corrected using the content of rRNA as a constant.

The variability of the protein allergens produced by this fungi has been often noted.^{2,19-22} Since the great majority of the work in this field has been related to human immunologic responses to the mold, the general assumption made by most investigators was that species variability along with type of propagule (spore or mycelium)

and extraction procedure were the most important factors. Our results indicated that, at least for the specific allergen-related sequence used in this study, species variability may not be as extensive as previously thought. This left the prospect that differing materials produced by the fungi were related primarily to differing extraction techniques and varying proteolytic degradation.

The comparison of the relative mRNA levels with the relative levels of measured allergen supported the conclusion that posttranslational events had great impact on extract composition. The fungi as a group are known to be prolific producers of unique proteolytic and glycolytic enzymes.^{10,13} We speculate that phenomena previously attributed to genetic variation were possibly a result of degradation during the commonly used allergen extraction process. Better understanding of the factors involved in these processes may result from further studies into the unique nature of fungal degradative enzymes.

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