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Purification, Identification, and cDNA Cloning of Jun a 2, the Second Major Allergen of Mountain Cedar Pollen

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The second major allergen of *Juniperus ashei* (mountain cedar) pollen, Jun a 2, has been purified and its cDNA cloned. The purified protein has a molecular mass of 43 kDa and its N-terminal 9-residue amino acid sequence is highly homologous to those of Cry j 2 and Cha o 2, the second major allergen of *Cryptomeria japonica* and *Chamaecyparis obtusa* pollen, respectively. cDNA clones encoding Jun a 2 were isolated after PCR based amplification, and their nucleotide sequences were determined. The cDNA contains an open reading frame of 507 amino acid residues, and encodes a putative 54-residue signal sequence and a 453-residue intermediate, which releases a C-terminal fragment upon maturation. Three possible N-linked glycosylation sites and 20 cysteine-residues are found in the deduced amino acid sequence. The amino acid sequence of Jun a 2 shows 70.7 and 82.0% identity with those of Cry j 2 and Cha o 2, respectively. Immunological observations that IgE antibodies in sera of Japanese pollinosis patients bind not only to Cry j 2 and Cha o 2 but also to Jun a 2 strongly suggest that Jun a 2 is an allergen of mountain cedar pollen, and that allergenic epitopes of these three allergens are similar. © 2000 Academic Press

Key Words: **Jun a 2; cDNA cloning; pollen allergen; specific IgE; pollinosis; mountain cedar.**

Plant pollen is one of the most common causes of seasonal allergic disease. In Japan, the prevalence of Japanese cedar (*Cryptomeria japonica,* Sugi) pollinosis has been increasing year by year, and this has become a serious social problem. Sato *et al.* reported that about 50% of young adults residing in the Tokyo area have specific IgE antibodies reacting to Japanese cedar pollen (1). Yasueda *et al.* have purified a major allergen of Japanese cedar pollen and designated it as Sugi basic protein (SBP) (2). The main component of the SBP is a basic glycoprotein with a molecular mass of 41–45 kDa as analyzed by means of SDS–PAGE under non-reducing conditions. After that, a second major allergen with a molecular mass of 37 kDa under non-reducing conditions was reported (3). These allergens were designated as Cry j 1 and Cry j 2, respectively, according to the WHO allergen nomenclature (4). We and others have previously isolated cDNA clones coding for these two allergens, and demonstrated that human IgE from pollinosis patients have reacted not only to native but also to recombinant Cry j 2 protein produced in *E. coli* (5–8).

The pollen of Japanese cypress (*Chamaecypris obtusa,* Hinoki) is also a well known airborne spring allergen in Japan. Recently, we have purified two major allergens (Cha o 1 and Cha o 2) from pollen of *C. obtusa,* and demonstrated that pollinosis patients have specific IgE antibodies to purified native Cha o 1 and Cha o 2 (9, 10). Furthermore, we have isolated cDNA clones encoding these two major allergens, and revealed that Cha o 1 and Cha o 2 were highly homologous with Cry j 1 and Cry j 2, respectively, at the amino-acid sequence level (9, 10). These findings are consistent with the clinical observation that many Japanese patients with pollinosis have specific IgE antibodies not only to *C. japonica* but also to *C. obtus* (11, 12).

On the other hand, pollen of mountain cedar (*Juniperus ashei*) is cross-reactive with the sera from patients allergic to Japanese cedar (13, 14), and is a major cause of allergen hypersensitivity in the United States (13). More recently, Midoro-Horiuti *et al.* (14–16) have isolated the two major extractable proteins of mountain cedar pollen, designated as Jun a 1 and Jun a 3, and cloned their cDNA. Though Jun a 1 was highly homologous to Cry j 1 and Cha o 1, Jun a 3 protein had an apparent molecular mass of 30 kDa on SDS–PAGE and bore no homology to previously described pollen allergens (16). Therefore, it was expected that the second major allergen(s) relating to Cry j 2 and Cha o 2 might also exist in the pollen of *J. ashei.* We examined

The nucleotide and amino acid sequences of Jun a 2 have been submitted to the EMBL/GenBank databases under the Accession No. AJ404653.

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the presence of related allergen(s) using rabbit polyclonal antibodies raised against recombinant Cry j 2 or Cha o 2 expressed in *E. coli* (6, 10). By the Western blot analysis of pollen extract, a Cha o 2 related allergen was detected.

In this paper, we report the purification of the second major allergen of *J. ashei* pollen, designated as Jun a 2, and the molecular cloning of cDNA. We also present evidence that IgE from all of the examined Japanese patients suffering from pollinosis binds to purified native Jun a 2. The primary structure of Jun a 2 that we report here should be useful in the determination of T-cell epitopes, as well as for developments of diagnostic methods and even a cure for pollinosis by *J. ashei*.

MATERIALS AND METHODS

Plant materials. Mountain cedar pollen was kindly donated by Dr. Goldblum (UTMB, Galveston, TX) and stored at −80°C until use.

Purification of Jun a 2 protein. Thirty grams mountain cedar pollen (*Juniperus ashei*) were defatted several times with 10 vol of diethylether and air-dried. The defatted pollen was suspended in 1,000 mL of 125 mM $NaHCO_3$ buffer (pH 8.3) containing 1% Triton X-100, and disrupted by sonication in an ice bath. The pollen proteins were extracted by stirring overnight at 4°C, and a crude Jun a 2 extract was obtained by centrifugation for 30 min at 22,000*g*. The extract was passed through a 120 mL bed vol of DEAE-cellulose column (DE-52, Whatman, UK) and the non-adsorbed material was collected. After adding solid NaCl to a final concentration of 0.5 M and adjusting the pH to 7.0, the DE-52 pass-through fraction was applied to a 50 mL bed vol of a zinc-chelating column (Amersham Pharmacia Biotech, Sweden). After extensive washing with 0.5 M NaCl/50 mM Tris–HCl (pH 7.0), Jun a 2 protein was eluted with 50 mM sodium acetate buffer (NaOAc, pH 4.0) containing 0.5 M NaCl. The eluate from the zinc-chelating column was dialyzed overnight against 20 mM sodium phosphate buffer (PB, pH 7.2) containing 2 M ammonium sulfate, and purified with a Phenyl Sepharose HiTrap HP column (Amersham Pharmacia Biotech, Sweden) with a reverse linear gradient of ammonium sulfate concentration from 2 M to 0 M in 20 mM PB (pH 7.2). The eluate fractions containing Jun a 2 protein were combined and dialyzed overnight against 10 mM NaOAc (pH 4.0). The dialysate was further purified using Mono S cation exchanging column chromatography (Amersham Pharmacia Biotech, Sweden). The elution was performed with a 0 to 1.0 M NaCl linear gradient in 10 mM NaOAc (pH 4.0), and Jun a 2 protein was purified in the fractions between 0.55 M and 0.75 M NaCl. The fractions containing Jun a 2 protein were pooled, concentrated with a Centricon YM-10 (Millipore, MA), and finally dialyzed overnight against PBS (phosphate buffered saline) at 4°C. The protein concentration of the dialysate was determined with a BCA Protein Assay Reagent (Pierce, IL).

The purity of Jun a 2 protein in the final preparation was analyzed by SDS–PAGE under reducing conditions, followed by silver staining of the gel. The yield of Jun a 2 protein was about 100 micrograms from 30 grams of mountain cedar pollen.

Detection of Jun a 2 protein by Western blotting and EIA. A crude extract of mountain cedar pollen, column fractions obtained during the purification steps or purified Jun a 2 protein were subjected to SDS–PAGE under reducing conditions, and electro-transferred to a PVDF membrane (Millipore, MA) using 25 mM Tris–HCl/192 mM glycine/0.1% SDS/20% methanol (pH 8.3) as a transfer buffer (17). After blocking overnight with 3% BSA and 1% NP-40 in PBS, the membrane was incubated for 2 h with 5,000-fold diluted anti-Cha o 2 rabbit sera. After washing several times with PBST (0.1% Tween-20 in PBS), the membrane was incubated for 1 h with 5,000-fold diluted horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, UK). After washing several times as above, Jun a 2 protein was detected with an Enhanced Chemi-Luminescence System (ECL, Amersham Pharmacia Biotech, UK).

In some cases, 10,000-fold diluted rabbit sera raised against recombinant Jun a 2 protein was also used to confirm the immunological cross-reactivity between Cha o 2 and Jun a 2.

For EIA detection of Jun a 2 protein during the purification process, 96-well microtiter plates (Nunc CovaLink, NY) were coated overnight with 0.1 mL aliquot of diluted column fractions at 4°C. After blocking for 2 h with 3% BSA and 1% NP-40 in PBS at room temperature, the wells were incubated for 2 h with anti-Cha o 2 rabbit sera and then 1 h with HRP-conjugated anti-rabbit IgG at room temperature. Jun a 2 protein was detected using TMB (3,3′,5,5′,-tetramethylbenzidine, DAKO, CA) as an HRP substrate.

Separation of Jun a 2 protein by gel electrophoresis. To analyze using SDS–PAGE, an aliquot of protein sample was mixed with a half volume of thrice-concentrated Laemmli's sample buffer, then heat-denatured and resolved by 12.5% gel electrophoresis according to the established method (18).

In order to compare the pI value of Jun a 2 with those of Cry j 2 and Cha o 2, two-dimensional gel electrophoresis was performed according to the method of O'Farrell (19). An aliquot of purified protein was separated in a 4% disk gel by isoelectric points in the presence of 8 M urea, 2% NP-40 and 2% Ampholine (pH 3.5–10, Amersham Pharmacia Biotech, Sweden). After electric focusing, the disk gel was placed on a 12.5% slab gel and resolved by SDS–PAGE as described above. The protein spot was detected by silver staining, and the pI value of each protein was determined.

N-terminal amino acid sequencing of the Jun a 2 protein. The purified Jun a 2 protein was subjected to SDS–PAGE and electro-transferred to a PVDF membrane similar to a Western blotting analysis, except that 10 mM CAPS/10% methanol (pH 11.0) was used as a transfer buffer (20). The membrane was rinsed 5 times with water, stained with Coomassie Brilliant Blue R-250, and Jun a 2 band was excised from the membrane for the direct protein microsequencing (HP G1005A Protein Sequencing System).

Isolation of RNA and synthesis of double-stranded cDNA. Total RNA was isolated from *J. ashei* pollen by the phenol-extraction methods as previously reported (5). Single-stranded cDNA primed with oligo(dT) or random hexamer was made from the total RNA with SuperScript RNase H(−) M-MLV reverse transcriptase (Gibco-BRL, MD, USA). RNA was then hydrolyzed by treating with alkali. Single-stranded cDNA and 5′-primer (1 pmol) were annealed, and the second strand was synthesized with the Klenow fragment of DNA polymerase by incubating initially for 10 min at 20°C, then for 10 min at 37°C.

Cloning of DNA fragment encoding the Jun a 2 protein by PCR amplification. PCR amplification was carried out in a DNA Thermal Cycler (PE-Biosystems, CA) with 25–35 cycles of 15 s at 96°C, 30 s at 55°C, and 90 s at 72°C. The reaction mixture (100 μL) contained 20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 25 mM KCl, 0.05% Tween 80, 100 μg/mL gelatin, 50 mM dNTPs, 1–2 units Taq DNA polymerase and 20 pmol of both primers, and was sealed with mineral oil. PCR products were analyzed by electrophoresis in 8% polyacrylamide or 1–2.5% agarose gel, and stained with ethidium bromide. DNA was extracted from polyacrylamide gel with 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS for 4–16 h at 37°C. DNA in the extract was concentrated by 2-buthanol extraction and precipitated by ethanol. DNA in the agarose gel was purified with a QIAEX II gel extraction kit (QIAGEN, Germany).

DNA fragments modified with restriction enzymes were ligated to appropriate pUC vector arms, and transfected into MC 1061 *E. coli* competent cells.

3′ RACE (rapid amplification of cDNA ends) was carried out following the method of Frohman *et al.* (21). We synthesized a $(dT)_{17}$-adaptor (5′-CTGCAGCGGCCGCTCGAGATCGATTTTTTTT-TTTTTTTTT-3′) and an adaptor (5′-CCATCTGCAGCGGCCGC-TCGAGATC-3′). The 3′ amplification primer was 5′-ggtagtcgac-AAGTCGCTACCTGTG-3′, where the sequence in capital letters matches that of Jun a 2 cDNA. Total RNA was reverse transcribed using the $(dT)_{17}$-adaptor as a primer. The cDNA was extracted with phenol/chloroform and diethylether, and then precipitated with ethanol. The 3′-end of Jun a 2 cDNA, about 500 bp in length, was amplified from the cDNA by 30–35 thermal cycles, using the adaptor and the 3′ amplification primer. The reaction mixture was subjected to agarose gel electrophoresis, and the DNA band with the expected mobility was excised. The cDNA fragment was digested with Not I and Sal I, and cloned into a pUC-type vector.

Double-stranded plasmid DNA with a cDNA insert was sequenced using an AmpliCycle sequencing kit (PE-Biosystems, CA) and [α-^{32}P] dCTP.

Production of recombinant Jun a 2 protein in E. coli. By splicing five Jun a 2 cDNA fragments, we constructed a cDNA sequence encoding residues 56-507 in Fig. 3, and bordered with Bam HI and Hind III restriction sites on pUC 19. The cDNA sequence was transferred onto an expression vector pQE9 (QIAGEN, Germany) and transfected into a host cell, *E. coli* M13 (pREP4). The recombinant cells thus obtained produce histidine-hexamer-tagged Jun a 2 protein upon induction with IPTG. The His-tagged Jun a 2 protein produced in *E. coli* was purified by Ni-NTA agarose column chromatography under denaturing conditions according to the manufacturer's instructions (QIAGEN, Germany).

Rabbit antibody against Jun a 2 was obtained by immunizing the recombinant His-tagged Jun a 2 protein to NZW female rabbits.

Fluorometric assay for IgE bound to native Jun a 2 protein. Sera from eight pollinosis patients (measured by AlaSTAT) caused by *C. obtusa* and from three non-allergic individuals were used. All of the donors consented to our study. Plasma of a mountain cedar pollinosis patient (PlasmaLab International, WA) was also used as a positive control. 96-well black plates (Dainippon Pharmaceutical, Osaka, Japan) were coated with one microgram of purified native Cry j 2, Cha o 2 or Jun a 2 protein for 16 h at 4°C, and blocked with Block Ace (Snow Brand, Sapporo, Japan) for 2 h at 37°C. Human serum was diluted 10-fold with Block Ace, transferred to each well and incubated for 4 h at 37°C. The amounts of IgE bound to the solid-phase antigen were evaluated by a fluorescence-based detection method, as described elsewhere (6).

RESULTS AND DISCUSSION

Purification and Characterization of Jun a 2

Recently, we have purified the second major allergen of *C. obtusa* pollen using anti-Cry j 2 rabbit sera and isolated its cDNA (10). Furthermore, it has been reported that the first major allergen of *J. ashei* pollen, Jun a 1, possessed a high degree of amino-acid sequence homology with Cry j 1 and Cha o 1 (14, 15). Therefore, in order to examine whether a Cry j 2 or Cha j 2 homologous protein(s) could be detected, we carried out immunoblot analyses of the extract of *J. ashei* pollen using anti-Cry j 2 and anti-Cha o 2 rabbit sera which were obtained by immunization of the recombinant proteins (6, 10).

A single band of 43 kDa was detected by immunoblot analyses with 1,000-fold diluted anti-Cry j 2 and anti-Cha o 2. Since the reactivity of anti-Cha o 2 was stronger than that of anti-Cry j 2, we used anti-Cha o 2 sera to detect and characterize the Cha o 2-related protein, and tentatively designated this as Jun a 2 (data not shown). To isolate the Jun a 2 protein, we adopted the same strategy as with the case of Cha o 2 purification (10). The crude extract of pollen was passed through a DEAE-cellulose column, and the non-adsorbed fraction was sequentially purified by zinc-chelate chromatography, Phenyl Sepharose hydrophobic chromatography and Mono S cation ion-exchange chromatography. In the step of zinc-chelate chromatography, the Jun a 2 protein adsorbed onto the resin at pH 7.0, while the Jun a 1 did not bind, and was eluted at pH 4.0. This was confirmed by immunoblot detection with anti-Cha o 1 or anti-Cha o 2 antibodies (data not shown) (9). From the result of zinc-chelate chromatography, it was suggested that Jun a 2 contained more histidine and cystein residues than those in Jun a 1, as with the case of Cha o 1 and Cha o 2.

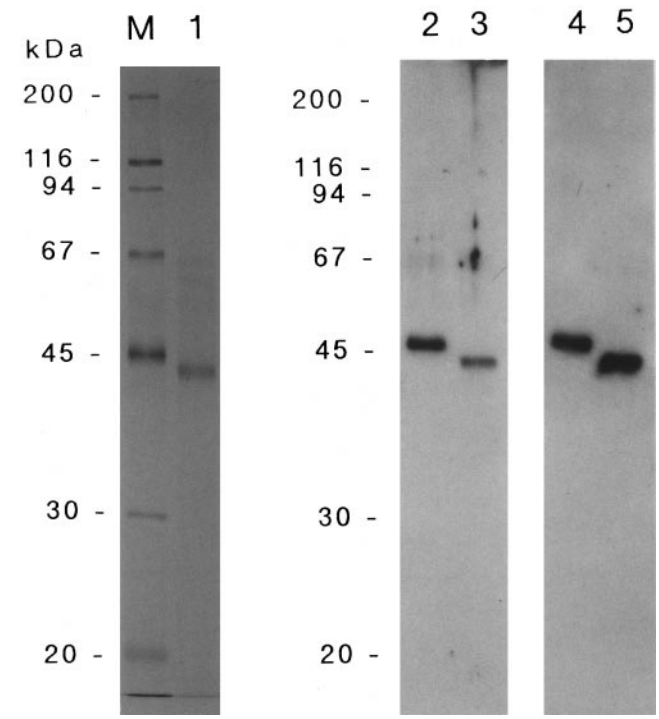

FIG. 1. Detection of Jun a 2 protein by silver staining and Western blotting. 100 ng of purified Jun a 2 was subjected to 12.5% SDS–PAGE under reducing conditions and detected by silver staining (lane 1). Similarly, 100 ng of purified Cha o 2 (lanes 2 and 4) or 100 ng of purified Jun a 2 (lanes 3 and 5) were subjected to SDS–PAGE, transferred to PVDF membrane. The Cha o 2 and Jun a 2 proteins on the membrane were detected by immunoblotting with anti-Cha o 2 rabbit serum (lanes 2 and 3) or anti-Jun a 2 rabbit serum (lanes 4 and 5) using ECL detection system, respectively.

The purity of Jun a 2 was verified by SDS–PAGE and silver staining (Fig. 1, lane 1). Western blot analysis revealed that both Cha o 2 and Jun a 2 were recognized by anti-Cha o 2 sera (Fig. 1, lanes 2 and 3). Rabbit sera raised against recombinant Jun a 2 also contained

antibodies which recognize both proteins (Fig. 1, lanes 4 and 5). Starting with 30 g of *J. ashei* pollen, we obtained about 0.1 mg of purified Jun a 2 protein, which was one fifth the amount of Cha o 2 protein purified from the equivalent amount of *C. obtusa* pollen (10).

The apparent molecular mass of purified Jun a 2 protein on SDS–PAGE was 43 kDa, which was similar to those of Cry j 2 and Cha o 2. The isoelectric point of Jun a 2 was estimated to be 8.55, whereas those of Cry j 2 and Cha o 2 were 8.25 and 8.65, respectively, as analyzed using the two-dimensional electrophoretic analysis of O'Farrell (data not shown). From these biochemical and immunological data, it was suggested that all of the three allergic proteins may belong to the same family.

The N-terminal amino acid sequence of Jun a 2 was determined to be Asp-Val-Ala-Ile-Val-Phe-Asn-Val-Glu. There is a similarity in the N-terminal amino acid sequences between Jun a 2 and Cha o 2 (7 of 9 amino acids were identical), and between Jun a 2 and Cry j 2 (5 of 9 amino acids were identical). Though most of the amino-acid sequence of signal peptides between Cry j 2, Cha o 2 and Jun a 2 was identical (only 5 in the first 50 residues were different each other, as described below), there was a difference, however, in the N-terminal position of mature Cry j 2, Cha o 2 and Jun a 2. This may be due to the different extraction conditions or the N-terminal heterogeneity of the mature protein (3, 8, 10). Alternatively, these differences between cedar and cypress may be due to the action of different signal peptidases during the secretion pathway.

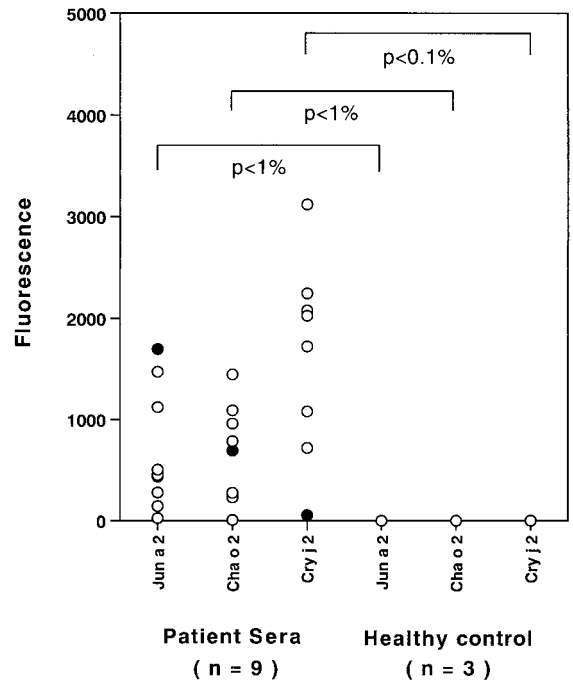

FIG. 2. Detection of specific IgE to purified Cry j 2, Cha o 2 and Jun a 2 in human sera. Sera from 8 Japanese cypress-allergic patients, from single mountain cedar-allergic patient (closed symbol) and from 3 non-allergic individuals were used, as described under Materials and Methods. Data are expressed as the means of duplicate measurements, and were statistically analyzed by the method of Aspin-Welch.

Biological and Clinical Significance of Jun a 2

To determine the allergenicity of Jun a 2, sera from patients allergic to mountain cedar and Japanese cypress were examined for binding of IgE to purified protein (6). All of the examined sera of pollinosis patients (8 sera from Japanese cypress-allergic patients and one serum from a mountain cedar-allergic patient) demonstrated enhanced binding to Jun a 2 protein, whereas 3 sera from healthy individuals showed no detectable reaction. However, the binding of IgE to Cry j 2 was higher than those to Cha o 2 and Jun a 2 in sera from Japanese patients (described below). On the other hand, serum IgE from the mountain cedar-allergic patient showed the highest binding to Jun a 2, a significantly high binding to Cha o 2, and weak binding to Cry j 2, respectively. Since the fluorescence values of negative IgE binding were below 5 in every case, we tentatively set the cut-off value to 10 relative fluorescence for positive and negative IgE binding in this immunological detection assay. A significant difference was observed in the fluorescence values between two groups of patients and healthy individuals for Jun a 2 (683 ± 600 vs 2.67 ± 0.58, $P < 1\%$), for Cha o 2 (638 ± 479 vs 2.33 ± 0.58, $P < 1\%$), and for Cry j 2 ($1{,}676 \pm 917$ vs 2.67 ± 1.16, $P < 0.1\%$), respectively. Therefore, it was suggested that high-titer sera from Japanese cypress-allergic patients also immunologically cross-react with mountain cedar pollen (Fig. 2).

Molecular Cloning of Jun a 2 cDNA and Its Nucleotide Sequence

Since anti-Cry j 2 rabbit sera cross-reacted with Jun a 2 protein, we assumed that there might also be significant homology between Jun a 2 and Cry j 2 at the gene level. We therefore synthesized some sense and antisense oligonucleotides based on the cDNA nucleotide sequence of Cry j 2 protein, and carried out RT-PCR experiments on mountain cedar pollen RNA. Three fragments of the expected sizes were amplified, cloned and sequenced. By combining the nucleotide sequences of the three cDNA fragments, we obtained about a half (820 bp) of Jun a 2 cDNA as a consensus sequence for multiple independent clones. The 3′-portion of cDNA (about 500 bp) was then obtained by the 3′-RACE method. Trials to obtain the 5′-portion of Jun a 2 cDNA by the 5′-RACE method were unsuccessful. However, we were able to obtain the 5′-portion of Jun a 2 cDNA by PCR using a sense primer based on

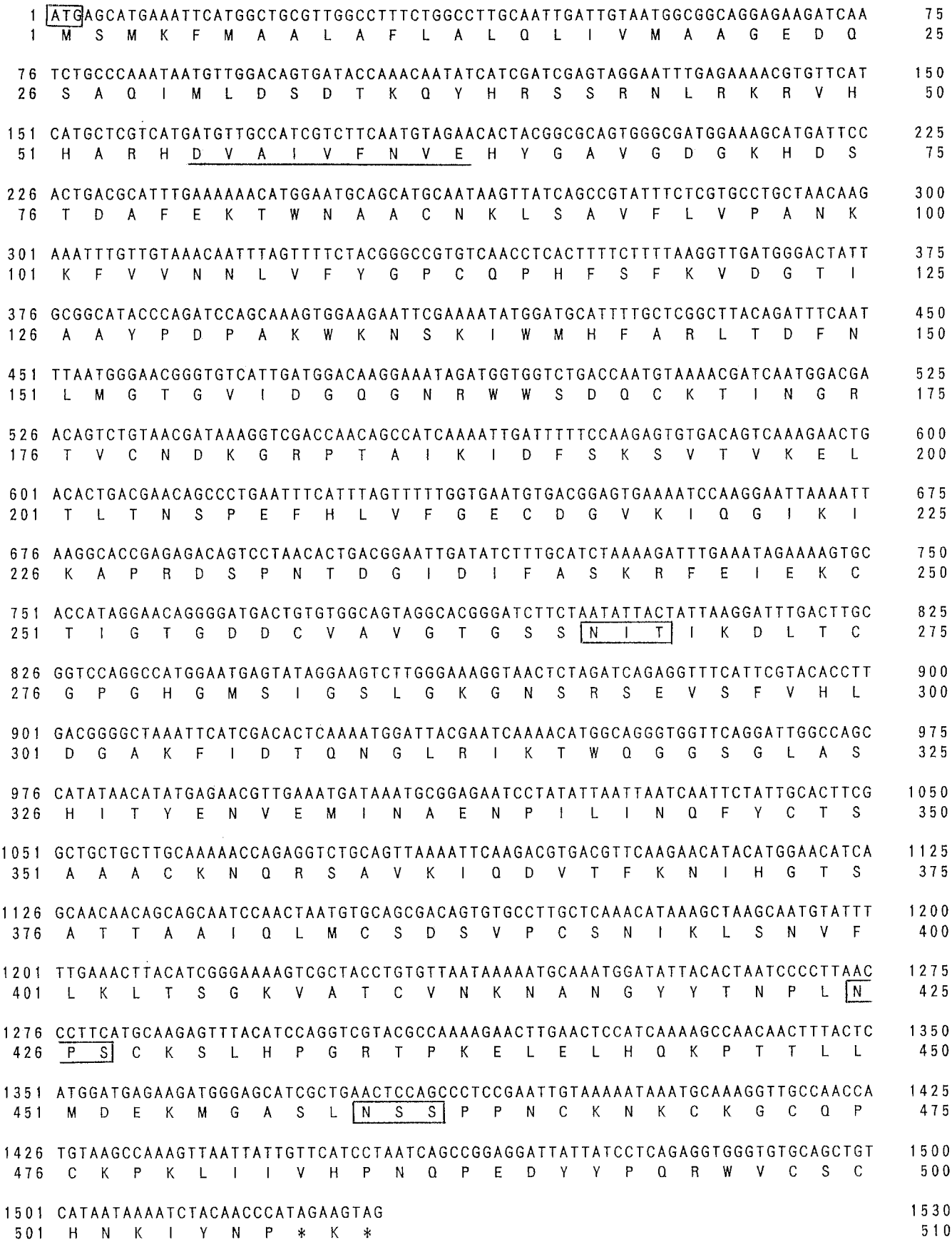

FIG. 3. Nucleotide and predicted amino acid sequences of Jun a 2 cDNA. An ATG initiation codon is boxed and a TAG stop codon is indicated by an asterisk. The underlined parts of the sequence is identical to the N-terminal amino acid sequence of purified mature Jun a 2 protein. Potential glycosylation sites are boxed.

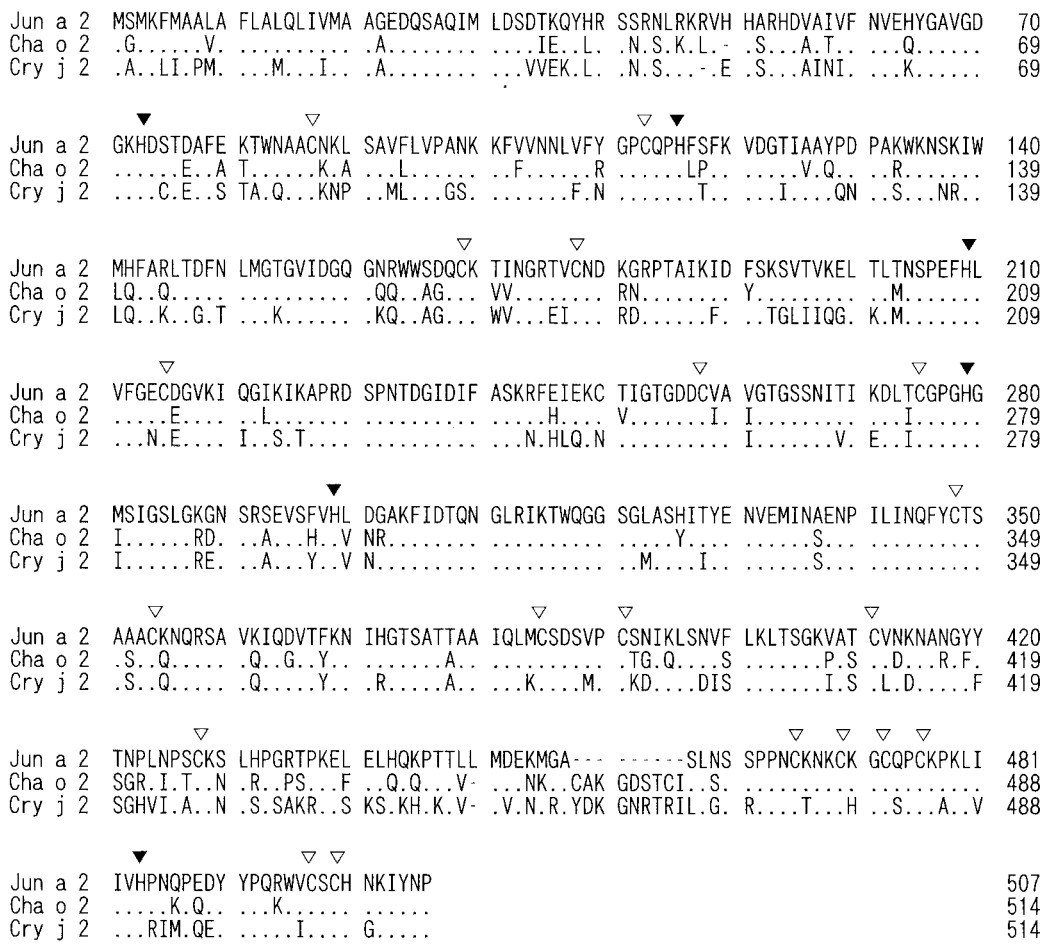

FIG. 4. Comparison of the deduced amino acid sequence of Jun a 2 with those of Cry j 2 and Cha o 2. The dots denote identical amino acids and the horizontal bars indicate blanks that have been inserted to maximize homology between them. Conserved 19 cystein and 6 histidine residues are indicated by open arrows and closed arrows, respectively.

the immediate upstream nucleotide sequence for the respective start codons of Cry j 2 or Cha o 2. By combining all of the sequence data, we determined the complete nucleotide sequence of Jun a 2 cDNA (1,759 bp). The sequence contained an open reading frame of 1,524 bp (Fig. 3). The deduced amino acid sequence of Jun a 2 protein was a 507-residue polypeptide. The amino acid sequence at position 55 through 63 from the initiator methionine completely matches the biochemically determined N-terminal amino acid sequence of purified Jun a 2 protein (Fig. 3, underlined), confirming the identity of the cDNA sequence obtained by RT-PCR.

Alignment of the amino acid sequence, as well as the cDNA sequence with those of Cry j 2 or Cha o 2 (6, 10), reveals 1-residue insertions near the N-terminal and another near the C-terminal, together with a 9-residue deletion near the C-terminal (Fig. 4). Except for these differences, Jun a 2 showed 70.7% identity with Cry j 2 and 82.0% identity with Cha o 2, respectively. This is consistent with the results of immunoblot analysis, in which anti-recombinant Cha o 2 rabbit sera showed a strong cross-reactivity with native Jun a 2 (Fig. 1), whereas rabbit antisera raised against recombinant Cry j 2 cross-reacted weakly with native Cha o 2 and Jun a 2, but strongly to native Cry j 2 (data not shown).

The N-terminal 54 amino acid residues are probably the signal peptide, because this amino acid sequence is not observed in the mature protein and is hydrophobic, as has been noted for other signal peptides. Therefore, Jun a 2 was found to consist of 453 amino acids and its predicted molecular mass to be 49,534 Dalton, whereas native Jun a 2 purified from pollen showed a band with the apparent molecular mass of 43 kDa under the reducing conditions (Fig. 1). These observations suggest that C-terminal processing of Jun a 2 may occur. Indeed, it has been reported that C-terminal 81 amino acid residues of Cry j 2 were cleaved from the precursor protein (8). The pre-sequence of 54 residues is longer than typical signal peptides, which usually consist of 20–30 hydrophobic amino acids. While the first 21 residues were certainly hydrophobic, the following 33

residues were rather hydrophilic. Since the hydrophilic stretch of the signal peptide is also observed in Cry j 2 and Cha o 2 precursors (6, 10), this stretch may have another unknown function.

Moreover, as we expected that Jun a 2 contained more cystein and histidine residues than those in Jun a 1 from the binding properties with the zinc-chelate column, Jun a 2 contained 20 cystein and 12 histidine residues. Interestingly, 19 of 20 sites for cystein residue were completely conserved between Jun a 2, Cha o 2 and Cry j 2 (Fig. 4) suggesting that all of the three allergens may possess a structurally similar conformation. This may also provide similar protein-chemical properties such as allergic and enzymatic activities.

We previously reported that Cry j 2 showed significant identities to polygalacturonases of tomato, avocado and maize (6). It has been reported that the histidine residue at position 278 of Cry j 2 may be a part of the active domain of polygalacturonase (22). The findings that the histidine residue at this position, as well as the positions of 19 cystein residues, were conserved between Cry j 2, Cha o 2 and Jun a 2 strongly suggest that Jun a 2 protein may also belong to the polygalacturonase family, and have an enzymatic activity which is involved in the cell wall degradation associated with pollen tube penetration and expansion into pistils.

The Jun a 2 protein also contained three potential N-linked glycosylation sites at 267-269, 425-427 and 460-462 corresponding to the consensus Asn-X-Ser/Thr motif (Fig. 3). Since the first one (Asn-Ile-Thr) of three motifs is conserved between Jun a 2 and Cha o 2, it is most likely to be a candidate for an N-glycosylation site.

In the present study, we demonstrated the purification and the molecular cloning of cDNA encoding Jun a 2 protein, the second major allergen of mountain cedar pollen. The high degree of sequence identity between this allergen and those of other conifers, such as Japanese cedar and Japanese cypress, may provide some common IgE epitopes among plants. A more complete characterization of the biological and immunological roles of shared structures among these allergens may help in an understanding of their allergenicity, and may also suggest new approaches for preventing and treating cedar pollinosis (23, 24).

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