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Pergamon

Molecular Immunology, Vol. 31, No. 6, pp. 491–498, 1994
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0161-5890/94 \$7.00 + 0.00

0161-5890(94)E0003-1

IMMUNOLOGICAL RELATIONSHIPS AMONG GROUP I AND GROUP V ALLERGENS FROM GRASS POLLEN

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(First received 3 July 1993; accepted in revised form 10 December 1993)

Abstract—Specific IgE antibodies have been affinity-purified from recombinant grass pollen allergens, and used to identify isoforms of the two major allergens of rye-grass pollen, *Lol p* I and *Lol p* V and cross-reactive allergens in other grasses. *Lol p* I-specific IgE (affinity-purified from the recombinant protein expressed by clone 13R which encodes amino acids 96–240 of *Lol p* I) identified four isoforms of the allergen. The same probe recognized cross-reactive epitopes in pollen proteins from 14 out of 16 grasses. The allergens identified by *Lol p* V-specific IgE (affinity-purified from the recombinant protein expressed by clones 12R or 19R which encode the full *Lol p* V protein) varied more in their physicochemical characteristics than the Group I isoforms. At least eight isoforms of *Lol p* V were identified by the *Lol p* V-specific IgE. The same probe recognized cross-reactive epitopes in pollen protein from 13 out of 16 grasses. Group I proteins were identified in grasses from two sub-families of the Poaceae, while the Group V allergens were only identified in pollen of grasses from one sub-family, the Pooideae.

Key words: *Lol p* I, *Lol p* V, IgE-binding, isoforms.

INTRODUCTION

Lol p I and *Lol p* V (which includes the allergens previously designated *Lol p* IX*) are the major allergens of rye-grass pollen. Together these allergens inhibit virtually all IgE binding to rye-grass pollen extract (Bond *et al.*, 1993). *Lol p* I and *Lol p* V are members of the Group I and Group V families of allergens, which are found in a number of grasses. The genes which encode both *Lol p* I (Perez *et al.*, 1990; Griffith *et al.*, 1991) and *Lol p* V (*Lol p* Ib, clone 12R; Singh *et al.*, 1991) have been cloned.

Proteins which are both antigenically and allergenically cross-reactive with *Lol p* I and *Lol p* V, have been identified in a number of grasses (Marsh *et al.*, 1970; Van Ree *et al.*, 1992; Singh and Knox, 1985; Standing *et al.*, 1987; Zhang *et al.*, 1991). MAbs have been used in conjunction with immunoblotting and ELISA to study antigenic cross-reactivity between allergens, especially among the Group I allergens (Standing *et al.*, 1987; Kahn and Marsh, 1986; Esch and Klapper, 1987). These techniques, however, reveal little information about overall homology between the proteins in question, and none about the nature of their allergenic epitopes.

IgE from sera of allergic individuals is a useful probe

for allergens but its polyclonal nature creates a number of problems. Individuals may be exposed to only one allergen, and from this exposure become sensitized to cross-reactive allergens in other grasses or they may have become sensitized through exposure to allergens from a number of different pollens. Techniques such as CRIE, RAST and ELISA inhibition have been developed to study allergenic cross-reactivity. These methods often use crude pollen extracts and the physical characteristics of the cross-reactive allergen are not determined. We have used a method where IgE against a single allergen can be isolated. In this method purified allergen in the solid phase is used to affinity purify IgE. In order to overcome the time-consuming and difficult task of obtaining pure allergens from pollen, IgE can be affinity-purified from fusion proteins of recombinant allergens. This allergen specific IgE can be used to study the distribution of allergenic epitopes in other grasses.

In this study we have used the fusion proteins of clones expressing *Lol p* I and *Lol p* V to isolate allergen specific IgE. *Lol p* I-specific IgE was affinity-purified from a fusion protein of a cDNA clone which expresses the C-terminal region of *Lol p* I and encompasses a major allergenic epitope (clone 13R; Griffith *et al.*, 1991). *Lol p* V-specific IgE was affinity-purified from the fusion proteins of two cDNA clones which express complete *Lol p* V proteins. There is an 80.4% similarity at the amino acid level between the proteins encoded by the two cDNA clones (clone 12R, Singh *et al.*, 1991; clone 19R; Ong *et al.*, 1993), and the proteins appear to be different isoforms of *Lol p* V.

The allergen specific IgE has been used to identify

*The Group V and Group IX allergens are now recognized to be members of the same allergen group. The International Union of Immunological Sciences nomenclature committee, has recommended that these allergens be called collectively Group V allergens. Thus the allergen originally designated *Lol p* Ib (Singh *et al.*, 1991) and later *Lol p* IX (Suphioglu *et al.*, 1992), now becomes *Lol p* V.

Table 1. Taxonomic relationship of clinically important grasses used in this study. This classification is taken from Watson (1990)

Family	Sub-family	Super-tribe	Tribe	Genus, Species	Common name
Poaceae	Pooideae	Triticoideae	Triticeae	<i>Agropyron cristatum</i>	Quack grass
			Bromeae	<i>Bromus inermis</i>	Smooth brome
		Poodae	Aveneae	<i>Agrostis alba</i>	Red top
				<i>Anthoxanthum odoratum</i>	Sweet vernal
			Pocae	<i>Avena sativa</i>	Cultivated oat
				<i>Holcus lanatus</i>	Velvet grass
				<i>Phalaris arundinacea</i>	Canary grass
				<i>Phleum pratense</i>	Timothy
				<i>Dactylis glomerata</i>	Orchard grass
				<i>Festuca elatior</i>	Meadow fescue
	Chloridoideae	Andropogonodae	Chloridoideae	<i>Lolium multiflorum</i>	Italian rye-grass
				<i>Lolium perenne</i>	Perennial rye-grass
				<i>Poa pratensis</i>	Kentucky bluegrass
				<i>Cynodon dactylon</i>	Bermuda
				<i>Sorghum halepense</i>	Johnson grass
				<i>Zea mays</i>	Maize

proteins which are allergenically cross-reactive with *Lol p* I and V in rye-grass and other grasses. These grasses were selected as sources of clinically important allergens, and according to their taxonomic group, representing three sub-families, three super-tribes and seven tribes within the grass family (see Table 1). Using this range of grasses we aimed to define the immunological relationships between Group I and V allergens from members of different taxonomic groups.

MATERIALS AND METHODS

Serum

Serum was collected by Dr R. Plomley at the Epworth Hospital (Richmond, Victoria, Australia), from patients with a history of allergy to grass pollen and a positive skin test to rye-grass or Bermuda grass pollen extract.

Pollen

Pollen was obtained from Greer Laboratories (NC, U.S.A.). The grasses used in this study were *Bromus inermis* (smooth brome), *Agropyron cristatum* (quack grass), *Secale cereale* (cultivated rye), *Dactylis glomerata* (orchard grass), *Festuca elatior* (meadow fescue), *Lolium perenne* (perennial rye-grass), *L. multiflorum* (Italian rye-grass), *Poa pratensis* (Kentucky bluegrass), *Avena sativa* (oat), *Holcus lanatus* (velvet grass), *Anthoxanthum odoratum* (sweet vernal grass), *Agrostis alba* (redtop), *Phleum pratense* (timothy grass), *Phalaris arundinacea* (canary grass), *Cynodon dactylon* (Bermuda grass), *Sorghum halepensis* (Johnson grass) and *Zea mays* (maize).

Isolation of soluble pollen proteins

Soluble proteins were extracted from pollen for two dimensional-polyacrylamide gel electrophoresis (2D-PAGE) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by shaking 100 mg of pollen in 1 ml of extraction buffer [15 mM phosphate buffered saline (PBS) (150 mM NaCl, 16 mM Na₂HPO₄,

4 mM NaH₂PO₄, pH 7.2), 1 mM phenylmethyl-sulfonyl fluoride] for at least 1 hr on ice. The mixture was spun at 12,000 g in a microcentrifuge for 5 min and the supernatant collected.

The concentration of proteins in the supernatant was determined using the Biorad assay (Biorad, C.A, U.S.A.).

Production of fusion protein and affinity purification of IgE

Lambda-gt11 containing clone 12R, 13R or 19R cDNA inserts, was used to infect *E. coli* strain Y1090 cell which were then plated onto Luria broth plates containing 100 µg/ml ampicillin. After 3 hr growth at 42 °C, the plates were overlayed with isopropyl-β-D-thiogalactopyranoside impregnated nitrocellulose filters (Hybond-C extra, Amersham, U.K.) and the incubation temperature reduced to 37 °C in order to induce the production of fusion protein. Four hours later, the filters were inverted and left for a further 4 hr. Two lifts were taken from each plate. These protein plaque lifts were incubated in 10% milk powder in PBS, to block unreacted binding sites.

After blocking, the protein plaque lifts were incubated in pooled sera from 11 allergic individuals, washed twice with PBS containing 0.1% Tween 20 (TPBS) and then twice with PBS only. The bound IgE antibodies were eluted from the plaque lifts by two 5 min incubations (with vigorous shaking) in 5 ml of 0.1 M glycine-HCl (pH 2.6)/1% BSA. The eluates were pooled and immediately neutralized using 2 M Tris-HCl (pH 9.0). IgE was also purified from plaque lifts of non-recombinant lambda-gt11 to use as a negative control. IgE affinity-purified from clone 12R, 13R and 19R plaque lifts was designated IgE-12R, IgE-13R and IgE-19R, respectively. The affinity-purified IgE was used to probe Western blots of pollen proteins separated by SDS-PAGE and 2D-PAGE.

Gel electrophoresis

SDS-PAGE. Protein samples (80 µg per lane) were denatured under reducing conditions by boiling for

3–5 min in the presence of 25 mM Tris-HCl, 2% dithiothreitol and 10% glycerol at pH 6.8. Proteins were then separated by SDS-PAGE using 10–15% gradient gels (with a 4% stacking gel) using the buffer system described by Laemmli (1970). Gradient gels were electrophoresed at 20 mA per gel for 1 hr, after which the current was increased to 30 mA per gel until the dye-front was 1 cm from the bottom of the gel. Gels were then stained with Coomassie Brilliant Blue R250 or left unstained for electrophoretic transfer.

2D-gel electrophoresis. Soluble rye-grass pollen proteins were separated in a mini-Protein II 2-D cell (Biorad, CA, U.S.A.) according to the manufacturers instructions. Proteins were diluted 1:1 in first dimension sample buffer (9.5 M urea, 2% CHAPS, 5% mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte). Thirteen µg of protein was applied per gel and the sample overlayed with first dimension sample overlay buffer (9.5 M urea; 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte; bromophenol blue). The first dimension gel was run for 3.5 hr with upper chamber buffer of 20 mM NaOH and lower chamber buffer of 10 mM H₂PO₄. The second dimension gel

was run for 45 min on 12% SDS-polyacrylamide gels as described above. The second dimension gel was overlayed with SDS sample equilibration buffer (62.2 mM Tris-HCl, pH 6.8; 2.3% [w/v] SDS; 5% [v/v] β-mercaptoethanol; 10% [w/v] glycerol; bromophenol blue). Gels were silver stained to reveal the protein profile.

Immunoblot analysis

Proteins separated by 2D-PAGE and gradient SDS-PAGE were transferred to nitrocellulose (BA 0.45 µm, Schleicher and Schuell, Dassel, Germany) according to Towbin *et al.* (1979). Proteins from 2D-gels were transferred at 200 mA for 3 hr, and gradient gels at 120 mA overnight. After protein transfer, unreacted binding sites were blocked by incubation of the nitrocellulose filters in powdered milk (10% in PBS).

The Western blots were incubated (overnight at room temperature) in sera from allergic individuals (1/4 dilution in PBS/BSA), or in affinity-purified IgE (undiluted) overnight at room temperature. IgE binding was visualized by incubation of the blot in ¹²⁵I-labelled anti-human IgE (Kallestad, TX, U.S.A.) (1/7 dilution in

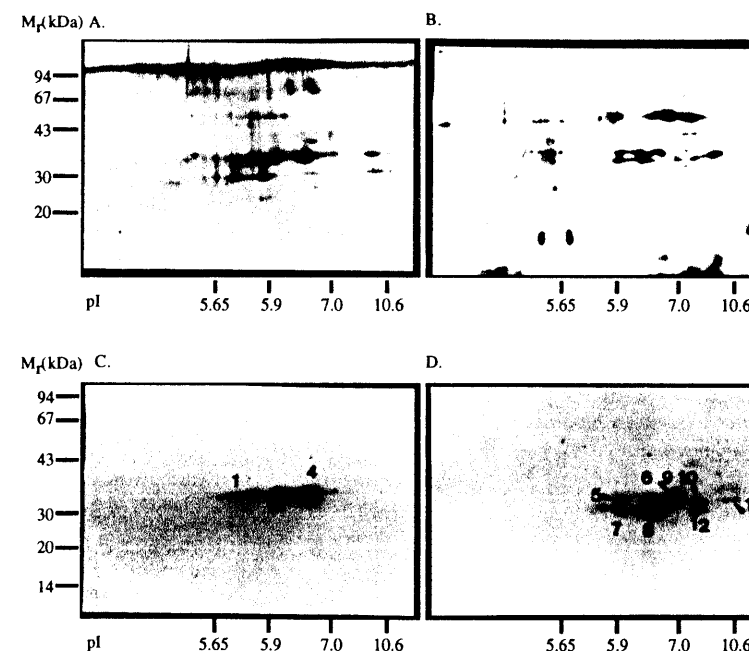


Fig. 1. Specificity of IgE affinity-purified from recombinant *Lol p* I and *Lol p* V for rye-grass pollen proteins separated by 2D-PAGE. Soluble rye-grass pollen proteins were separated by 2D-PAGE and either (A) silver stained with Coomassie Brilliant Blue R250 or transferred to nitrocellulose and the Western blots screened with (B) IgE from pooled sera of individuals allergic to rye-grass; (C) IgE-13R; and (D) IgE-12R. M_r (kDa) is denoted at the left and pI below. Components which bound IgE-13R or -12R have been numbered.

PBS/BSA) overnight at room temperature, followed by exposure to X-ray film (Kodak XAR X-ray film) at -70°C with an intensifying screen for 24 hr.

RESULTS

Identification of cross-reactive isoforms of *Lol p 1* and *Lol p V*

The recombinant allergens expressed by clones 12R, 19R or 13R were used as a solid matrix to affinity purify allergen specific IgE from pooled sera of individuals allergic to rye-grass pollen.

To identify isoforms of Group I and V allergens which differ in pI and M_r , 2D-electrophoresis was used. Figure 1B shows binding of IgE from pooled allergic sera to proteins from crude rye-grass pollen extract separated by 2D-PAGE. IgE affinity-purified from the 13R recombinant protein (IgE-13R) bound to four 35 kDa proteins with acidic to neutral pI (bands numbered 1–4 in Fig. 1C, Table 2). These are the major proteins on silver stained gels and are considered to be isoforms or variants of *Lol p 1*. IgE affinity-purified from the 12R recombinant protein (IgE-12R) recognized eight allergenic components 28–32 kDa which were considered to be isoforms of *Lol p V* (bands numbered 5–12 in Fig. 1D, Table 2). Binding of IgE affinity-purified from the 19R recombinant protein (IgE-19R) was identical to that of IgE-12R (results not shown).

Identification of allergenically cross-reactive proteins in pollen from a number of grasses

As the IgE affinity-purified from the recombinant *Lol p 1* and *Lol p V* had been shown to recognize natural isoforms of both *Lol p 1* and V, these antibodies were used to probe pollen proteins from other grasses in order to identify proteins which share allergenic epitopes with the recombinant *Lol p 1* and V.

Table 2. Binding of affinity-purified IgE to rye-grass pollen proteins showing approximate M_r and pI of the allergens identified

Allergen no.	M_r (kDa)	pI	IgE binding ^a	Group
1	35	5.5	IgE-13R	I
2	35	5.9	IgE-13R	I
3	35	6.45	IgE-13R	I
4	35	7	IgE-13R	I
5	32	6	IgE-12R/19R	V
6	32	6.45	IgE-12R/19R	V
7	30	6	IgE-12R/19R	V
8	30	6.45	IgE-12R/19R	V
9	30	7.2	IgE-12R/19R	V
10	30	8.3	IgE-12R/19R	V
11	30	>10	IgE-12R/19R	V
12	28	8.0	IgE-12R/19R	V

^aIgE-13R: IgE affinity-purified from recombinant *Lol p 1* (clone 13R fusion protein); IgE-12R/19R: IgE affinity-purified from recombinant *Lol p V* (clone 12R or 19R fusion protein).

Screening of a Western blot of pollen proteins from different grasses with pooled sera from individuals allergic to rye-grass pollen, showed that all grasses contained a number of allergens (Fig. 2B). IgE-13R, purified from recombinant *Lol p 1*, bound to two proteins in rye-grass pollen extract. Due to the excess protein loaded on the gel, the upper band is visible as a smear of 35–42 kDa. Where less protein is loaded this band is resolved as a single 35 kDa protein (results not shown). Binding of IgE-13R to the second 29 kDa protein is less intense. IgE-13R also recognized proteins in 14 of the 16 grasses studied (Fig. 2C). Strong binding of IgE-13R was observed to pollen proteins with approximate size of 35 kDa from grasses of the super-tribe Pooideae (sub-family Pooideae; including orchard grass, meadow fescue, perennial rye-grass, Italian rye-grass, Kentucky blue-grass, velvet grass, sweet vernal, redtop, timothy and canary grass). These proteins correspond in M_r with the proteins stained most strongly by Coomassie Brilliant Blue (Fig. 2A). Weak binding of IgE-13R to pollen proteins from quack grass, smooth brome (sub-family Pooideae; super-tribe Triticoideae), Johnson grass and maize (sub-family Panicoideae; super-tribe Andropogonoideae) was observed. No binding was detectable to pollen proteins from cultivated oat (sub-family Pooideae; super-tribe Pooideae) and Bermuda grass (sub-family Chloridoideae).

IgE-12R bound to proteins in 13 of the 16 grasses tested, namely smooth brome, quack grass, orchard grass, meadow fescue, perennial rye-grass, Italian rye-grass, Kentucky bluegrass, cultivated oat, velvet grass, sweet vernal grass, redtop, timothy and canary grass. All these grasses are members of the sub-family Pooideae. The signal intensity for IgE-12R was highest for proteins in four grasses; perennial and Italian rye-grass, orchard grass and meadow fescue (sub-family Pooideae; super-tribe Pooideae; tribe Poeae) (Fig. 2D). Meadow fescue appears to have four cross-reactive IgE binding components of 28–32 kDa, while perennial and Italian rye-grass both have three components. In Kentucky bluegrass only one 30 kDa component bound to IgE-12R.

The IgE-12R bound to one or two components in the size range 28–35 kDa in pollen extracts from smooth brome, quack grass (sub-family Pooideae, super-tribe Triticoideae, tribe Triticeae), red top, sweet vernal, velvet grass, timothy and canary grass (sub-family Pooideae; super-tribe Pooideae; tribe Aveneae). The components identified in cultivated oat are of higher M_r than those found in the other grasses.

An additional, 45 kDa allergen was recognized by IgE-12R in pollen extracts from perennial and Italian rye-grass, meadow fescue and canary grass. Binding of IgE-19R was identical to that of IgE-12R (results not shown).

DISCUSSION

This study defines the immunologic relationships of Group I and Group V allergens from the pollen of different grasses using allergen specific IgE. Recombinant

fusion proteins have been used to affinity-purify allergen-specific IgE antibodies and these antibodies identify conserved IgE epitopes on natural isoforms of both allergen groups. This ability to recognize different isoforms of the same allergen makes the affinity-purified IgE antibodies useful for identifying proteins which are cross-reactive with the recombinant allergens in the pollen from other grasses.

Group I allergens

To affinity-purify *Lol p 1*-specific IgE, we selected the fusion protein encoded by cDNA clone 13R. This fusion protein has been shown to bind IgE (Griffith *et al.*, 1991) and includes the IgE epitope identified by Esch and

Klapper (1989) which is found at the C-terminal end of *Lol p 1* (Perez *et al.*, 1990; Griffith *et al.*, 1991). On 2D-immunoblots the *Lol p 1*-specific IgE recognized four isoforms of *Lol p 1*, all with the same M_r (35,000) but with pI values ranging between 5.5 and 7. These results confirm earlier biochemical findings, that *Lol p 1* has 3–4 isoforms (Johnson and Marsh, 1965) with differing pIs (Ansari *et al.*, 1987). These isoforms seem to result from microheterogeneity in the amino acid composition of proteins encoded by isogenic variants of a multi-gene family. Both Perez *et al.* (1990) and Griffith *et al.* (1991) have reported different cDNA isoforms of *Lol p 1* with minor amino acid differences. Some IgE reactive epitopes on these allergen isoforms appear to be highly

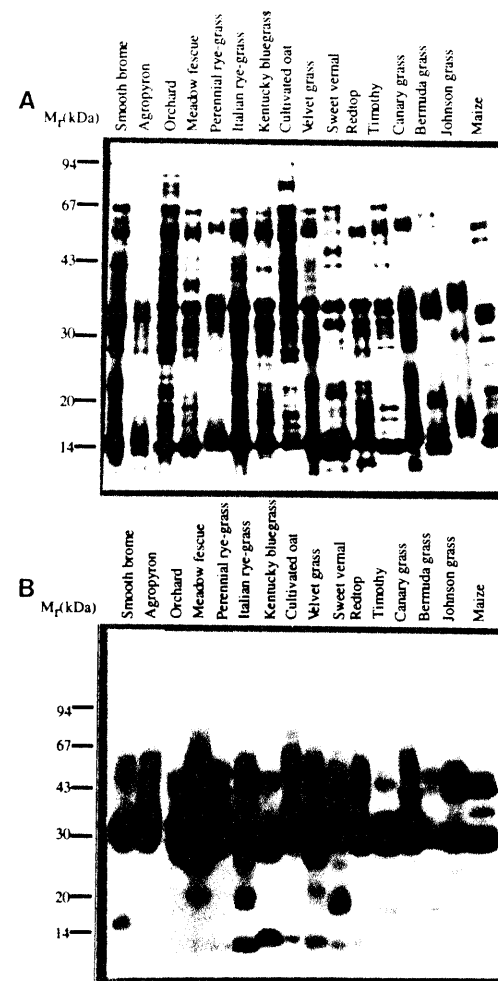


Fig. 2



SHORT COMMUNICATION

COMPLETE NUCLEOTIDE SEQUENCE OF A RAINBOW TROUT cDNA ENCODING A MEMBRANE-BOUND FORM OF IMMUNOGLOBULIN HEAVY CHAIN

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(Received 5 January 1994; accepted 15 February 1994)

Introduction: The molecular characterization of genes involved in the humoral and cellular immune response in fish is important in the understanding of immune development in vertebrate species. There have been several reports dealing with immunoglobulin heavy chain (IgH) genes from primitive taxa ranging from elasmobranchs (Kokubu *et al.*, 1988) to Atlantic salmon (Hordvik *et al.*, 1992). Recently, there have been two reports dealing with cDNAs encoding for complete secretory IgHs along with partial clones of membrane-bound forms of IgH in trout (Lee *et al.*, 1993 and Andersson and Matsunaga 1993). Polymerase chain reaction amplification (PCR) with degenerative primers were used to derive a rainbow trout VH probe that, in turn was used to screen a peripheral blood leukocyte (PBL) cDNA library from a single rainbow trout (*Oncorhynchus mykiss*), Shasta strain. Several full length secretory and membrane-bound forms of trout IgHs were isolated. Here we briefly describe the complete nucleotide sequence from one of the membrane-bound forms of trout IgH, clone RBTIGTM2.

Material and Methods: An alignment of amino acid and nucleotide sequences of several vertebrate VH genes were used as the basis for designing degenerative oligonucleotide primers which were as follows: Sense FR2=5'-CCISAIMGNCCTGYAMAGCCTCYGGITT-3' and antisense FR3=5'-CCGGATCCGCACAI TAATAAVNYGCGTGTCTC-3'. The FR3 primer incorporates the fact that most VH genes end in the amino acid motif of Y-Y-C-A-R. PCR was conducted using genomic trout DNA as the template. The resulting amplified product was cloned using a TA cloning kit (Invitrogen) and then sequenced for verification of identity. The VH gene clone was then radiolabeled using random hexamers (BRL) and used to screen a trout PBL cDNA library which had been constructed in a Uni-ZAPXRII vector system as per manufacture's instructions (Stratagene). The library was screened under high stringency conditions and several putative clones were isolated and characterized. Sequencing of the VH clone and full length IgH clones were accomplished using both manual and automated sequencing in both directions. Manual sequencing was performed using the Sequenase 2.0 system (USB) and automated sequencing was conducted using an ABI 373A sequencer located at the OSU Center for Gene Research and Biotechnology Central Services Facility. All sequence analysis was conducted using the Genetics Computer Group Sequence Analysis Software Package Version 7.2-UNIX.

Results and Discussion: Clone RBTIGTM2 encodes 1703 nucleotides, including an 18 amino acid leader peptide, VH region, CH1-3 domains and a TM domain followed by 3' UT sequences including a putative polyadenylation site (Fig 1). The nucleotide sequence of the RBT VH region was most similar to ladyfish (Amemiya and Litman 1990; ~81% identity). The CH1-3 domains were most similar to trout (Lee *et al.*, 1993; ~98% identity) and to Atlantic salmon (Hordvik *et al.*, 1992; ~95% identity). One notable difference was the addition of an extra codon (GCA) between the CH2-CH3 boundary which was not seen in other published reports of trout IgH cDNAs. The TM domain was completely conserved when compared to the other trout TM sequences. This clone adds further support to the unusual splicing patterns seen in other teleosts including Atlantic salmon (Hordvik *et al.*, 1992), Atlantic cod (Bengtén *et al.*, 1991) and catfish (Wilson *et al.*, 1990), where the entire CH4 domain was missing in the membrane bound forms of IgH. A second full length membrane bound IgH was sequenced and compared to RBTIGTM2. The second clone possessed a limited number of nucleotide differences in the constant domains (Fig 1) which resulted in 5 amino acid substitutions. Although this clone could conceivably be representative of another isotype, the limited amino acid variance suggests an allotypic variant of RBTIGTM2.

Acknowledgements: Funds for this research were derived from N.I.E.H.S grant # ES05783. Oregon State Technical Paper Number 10,400.

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