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GTP-binding proteins serve to couple cell-surface receptors to intracellular effectors. The molecular identities of G-proteins expressed in the olfactory epithelium of the freshwater channel catfish are being studied by immunoblotting and hybridization experiments.

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Significant advances in our understanding of the molecular events underlying olfactory reception have been made in the past few years (for reviews, see ref. 1-3). It is now generally, but not universally (4-7), accepted that the initial event of olfactory signaling involves the interaction of stimuli with macromolecular receptors localized in the membranes of the apical dendritic cilia. Stimulus binding to the receptors initiates the molecular events underlying the signal transduction process which transmits stimulus-encoded information across the plasma membrane. A variety of biochemical and electrophysiological evidence supports the hypothesis that olfactory signal transduction is mediated by GTP-binding regulatory proteins (G-proteins) that serve to link cell-surface receptors to intracellular effectors (8, 9). Initial biochemical studies with isolated cilia preparations demonstrated the potential involvement of both cyclic nucleotide and phosphoinositide-derived second messengers in olfaction (8). It is particularly interesting to note that these initial results have recently been confirmed using rapid kinetic methodology (10-12). These recent studies have shown that the rates of stimulus-induced formation of both cAMP and IP_3 (derived from phosphoinositide lipids) are sufficiently rapid to be consistent with electrophysiological latency measurements. These observations therefore indicate the likely involvement of these second messengers in regulation of the ion channels underlying membrane depolarization.

Although substantial evidence indicates the involvement of

G-protein-linked second messengers in vertebrate olfaction, the majority of currently available data was obtained using single, individually presented, stimuli. It is obvious however that an organism rarely, if ever, encounters a single stimulus by itself, but rather encounters a variety of stimulus mixtures with complex spatial and temporal characteristics (13). Essentially no data are currently available regarding the molecular mechanisms mediating olfactory responses to stimulus mixtures. However, given the biochemical and electrophysiological data supporting a role for G-protein-linked second messengers in olfactory transduction, it is likely that these second messengers also mediate responses to stimulus mixtures. In addition, a reasonable working hypothesis involves the assumption that regulatory interactions between these second messengers, perhaps via calcium/calmodulin (14), are involved in mediating responses to stimulus mixtures.

An important consideration for experimental approaches to understanding the molecular and cellular events underlying responses to stimulus mixtures involves the choice of an appropriate model system. We have chosen to study responses to stimulus mixtures in the olfactory system of the freshwater channel catfish (Ictalurus punctatus). From a practical viewpoint, this organism is a readily accessible, inexpensive, model system that provides large quantities of tissue for biochemical studies. In addition, the subtypes and specificities of the receptors have been characterized in detail by biochemical (15, 16) and neurophysiological (17, 18) assays. Binding studies, in

the presence and absence of guanine nucleotides, demonstrated that the amino acid chemoreceptors in this organism are functionally coupled to one or more G-proteins (19). Biochemical studies have also shown that stimulus amino acids, in the presence of GTP, evoke increases in both cAMP (20) and IP_3 (21, 22) in isolated cilia preparations. Caprio and coworkers have also shown in this model system that in vivo neurophysiological responses to binary and trinary stimulus mixtures can be predicted from the specificities of the receptor subtypes as defined previously by biochemical and electrophysiological criteria (23, 24). Thus, the channel catfish represents a unique and appropriate model system for molecular and neurophysiological studies of olfactory responses to stimulus mixtures.

Previous biochemical studies (15, 16) showed that at least four discrete receptor subtypes exist in the olfactory system of the catfish, in agreement with neurophysiological recordings (18). The functional interaction of these receptors with G-proteins coupling the receptors to adenylate cyclase and phospholipase C was also demonstrated in vitro (19, 21, 22). G-proteins were identified in isolated cilia preparations by immunoblotting with subunit-specific antisera and by bacterial toxin-catalyzed ADP-ribosylation (19). These experiments revealed the presence of two cholera toxin substrates (45 and 42 kDa) and a single pertussis toxin substrate (40 kDa) in the isolated cilia. At the time of these observations, the 45 kDa polypeptide was presumed to correspond to the alpha subunit of

G_s, based on its electrophoretic mobility and susceptibility to cholera toxin. The 42 kDa polypeptide was assumed to represent a proteolytic cleavage product of the 45 kDa cholera toxin substrate. Based on its molecular size and immunoblotting with subunit-specific antisera, the 40 kDa pertussis toxin substrate was shown to be distinct from G_o, a G-protein of unknown function that is predominantly expressed in brain. However, these studies did not provide definitive evidence to unambiguously clarify the molecular nature of the three toxin substrates. In addition, the recent molecular cloning of the olfactory neuron-specific G-protein, G_{olf}, from rat olfactory epithelium (25), has prompted a re-evaluation of the previous data regarding the identities of the G-protein alpha subunits in the isolated cilia. Establishing the molecular identities of the G-proteins in olfactory cilia is a necessary and critical prerequisite to elucidation of the molecular links between the receptors and second messenger responses to stimulus mixtures.

Materials and Methods

Materials. Polyclonal rabbit antisera to a peptide derived from rat G_{olf} was generously provided by Dr. F. Margolis of the Roche Institute for Molecular Biology. Polyclonal rabbit antisera to a peptide derived from G_s was generously provided by Dr. S. Mumby of the University of Texas. Polyclonal rabbit antisera to the C-terminal peptides of G_s and G_{olf} and to G_{i1} and G_{i2} were obtained from NEN. Peroxidase-conjugated anti-rabbit IgG $F(ab)_2$ fragments were obtained from Sigma. Westran and nitrocellulose

membranes were obtained from Schleicher & Schuell. TMB substrate and hydrogen peroxide solutions were obtained from Kierkegaard & Perry Labs. Reagents for isolation of poly(A)$^+$ RNA were obtained from Invitrogen and Gibco/BRL. Reagents for nonradioactive labeling of RNA transcripts were obtained from Boehringer-Mannheim. Restriction enzymes, agarose, competent cells and other reagents for molecular biology were obtained from Gibco/BRL. Plasmids encoding rat OMP and G_{olf} were generously provided by Dr. R. Reed of The Johns Hopkins University.

Affinity Purification of Polyclonal Golf Antibody. A peptide corresponding to amino acids 9-25 of rat G_{olf} (25) was coupled to bovine thyroglobulin with glutaraldehyde. Antisera to the peptide conjugate were obtained from two rabbits following multiple immunizations. An affinity column was prepared by coupling the peptide to CH-Sepharose (Pharmacia) according to the manufacturer's protocol. The column (2.5 ml) contained about 1 mg peptide/ml Sepharose. Affinity purification of G_{olf} antibody was performed as described previously (25, 26), and the purified antibody was stored at -80°C in small aliquots.

Immunoblotting. Isolated cilia preparations from rat and catfish were obtained as previously described (21). Membrane preparations were obtained from deciliated olfactory epithelium by homogenization of the tissue in the same buffer used for cilia preparations supplemented with 0.1 mg/ml aprotinin, 1 mM PMSF and 10 ug/ml leupeptin. Membranes were isolated following differential centrifugation as previously described for cilia prepara-

tions (21). Membrane fractions were separated by electrophoresis through 10% SDS-polyacrylamide gels and transferred to Westran membranes as described previously (19). Immunoblotting was performed as described previously (19), except that the blocking solution also contained 5% normal goat serum (Sigma) and wash buffers contained 0.05% Tween 20. Immunoreactive bands were visualized with TMB substrate and hydrogen peroxide.

Plasmid Purification. DH5 competent cells were transformed with plasmids encoding rat OMP and G_{olf} by established methodology (27). Transformation efficiencies averaged about 10^8 transformants/ug DNA. Transformed cells were plated on LB agar medium supplemented with 100 ug/ml ampicillin and grown at 37°C overnight. Single colonies were subcultured in LB broth supplemented with 100 ug/ml ampicillin and grown at 37°C overnight. The integrity of the plasmid DNA from these cultures was verified by restriction digestion and agarose gel electrophoresis following miniprep isolation of the DNA (28). Selected cultures were subsequently used to inoculate large-scale (200-300 ml) LB broth cultures. After overnight incubation, plasmid DNA was isolated by alkaline lysis and purified by cesium chloride-ethidium bromide gradient centrifugation (28) or by chromatography through Plasmid Quik columns (Stratagene). The integrity of the isolated plasmid DNA was verified by restriction digestion and agarose gel electrophoresis. Isolated plasmid DNA was stored in small aliquots at -20°C after determining the yield of DNA spectrophotometrically (1 A^{260} unit = 50 ug dsDNA/ml).

Isolation of Poly(A)$^+$ RNA. Rat and catfish tissues were excised as rapidly as possible after exsanguination and immediately frozen in liquid nitrogen. Tissues were stored up to two months in liquid nitrogen or at -80° C. Poly(A)$^+$ RNA was isolated by homogenization of the frozen tissue in Lysis Buffer (Invitrogen FasTrack) or in 4 M guanidinium isothiocyanate/25 mM sodium citrate, pH 7.0 (29) with a Polytron. The Polytron probe was soaked for 15 min in 3% hydrogen peroxide prior to use to prevent RNase degradation. Poly(A)$^+$ RNA was obtained by both methods by selection on oligo-dT cellulose according to standard methodology. Following ethanol precipitation, the yield of RNA was determined spectrophotometrically and the quality of the isolated material was evaluated by agarose gel electrophoresis.

Preparation of Labeled RNA and Oligonucleotide Probes. To obtain labeled RNA probes, plasmids were linearized with appropriate restriction endonucleases, treated with proteinase K, extracted with phenol/chloroform and ethanol precipitated. Labeled RNA probes were obtained by in vitro transcription using the reagents and protocol supplied by Boehringer Mannheim for nonradioactive labeling of RNA. Oligonucleotide probes for tubulin and actin were obtained from NEN and labeled with digoxigenin-11-dUTP by DNA tailing according the protocol supplied by Boehringer Mannheim.

Northern Blotting. Glyoxylated RNA preparations were resolved in 1.2% agarose gels and transferred to nitrocellulose membranes by overnight capillary transfer (30). After processing

of the membranes (30), prehybridization was performed at 42°C in 50% formamide/5xSSC/0.1% N-lauroylsarcosine/0.02% SDS/5% blocking reagent (Boehringer Mannheim) for 1 hr. After addition of heat-denatured labeled probes, overnight hybridization was performed in the same solution at 42° C. The blots were washed twice in 2xSSC/0.1% SDS at room temperature for 5 min and twice at 42° C with 0.1xSSC/0.1% SDS for 15 min. Labeled bands were visualized immunologically according to the protocol supplied by Boehringer Mannheim.

Results and Discussion

Immunoblotting of G-protein Alpha Subunits in Membrane Preparations from Olfactory Epithelium. The recent molecular cloning of a G-protein that is exclusively expressed within olfactory neurons (25) prompted a re-evaluation of the molecular identities of the two cholera toxin substrates previously identified in isolated cilia (19). Rat G_{olf} is a cholera toxin substrate (31) and functionally interacts with adenylate cyclase in a heterologous system (25). Since the olfactory amino acid receptors of the catfish are also linked to adenylate cyclase in a GTP-dependent manner (20), it is necessary to determine which of the two cholera toxin substrates in the cilia is coupled to adenylate cyclase. In addition, it is reasonable to assume that if G_{olf} represents the olfactory equivalent of retinal transducin, then it should be highly conserved across species. To test this hypothesis and to determine the identities of the cholera toxin substrates in isolated cilia, immunoblotting

studies using subunit-specific antibodies were performed. The amino acid sequences of the peptides used to obtain the three antibodies that were employed in the immunoblotting studies are shown in Table I. Isolated cilia and membranes from deciliated olfactory epithelium were prepared from rat and catfish. These preparations were then resolved in SDS-polyacrylamide gels, transferred to Westran mambranes and probed with each of the three antibodies described in Table I. Since the peptides used to obtain the antibodies were derived from mammalian amino acid sequences, rat cilia and deciliated membranes were used as a positive control. Immunoblots probed with affinity-purified antibody RR3, specific for rat G_{olf} (25), are shown in Fig. 1. Consistent with previous data (25), a 45 kDa band was labeled in both cilia and membranes from deciliated olfactory epithelium in the rat. This immunoreactivity was completely abolished by prior incubation of the antibody with the peptide (data not shown). However, no immunoreactive bands could be detected in cilia or deciliated membranes from catfish with this antibody. The same result was obtained using a variety of gel conditions and protein loadings. The absence of immunoreactivity in the catfish was somewhat surprising, as several peptide antisera to G-protein subunits cross-reacted in catfish in previous experiments (19). Two additional antibodies were therefore tested for immunoreactivity with the two cholera toxin substrates in catfish olfactory epithelium. Both the 45 and 42 kDa toxin substrates in isolated cilia and deciliated membranes from catfish were recognized by

antiserum 584, the most specific antiserum currently available for G_s (32). The same result was obtained with antiserum RM1, which recognizes the C-terminal decapeptide of both G_s and G_{olf} (Fig. 2). Our interpretation of these results is that the 42 kDa polypeptide corresponds to G_s, while the 45 kDa polypeptide represents the catfish homolog of G_{olf}. However, antibody cross-reactivity between G_s and G_{olf} (34) precludes definitive conclusions regarding these results.

The identity and tissue localization of the 40 kDa pertussis toxin substrate was also evaluated by immunoblotting using an antiserum to the C-terminal decapeptide of G_{i1} and G_{i2} (33). In isolated cilia and in membranes prepared from deciliated olfactory epithelium from both rat and catfish, a single band of 40 kDa was recognized by this antiserum (Fig. 3). These results, taken together with previous data (19), indicate that the 40 kDa pertussis toxin substrate corresponds to G_{i1} and/or G_{i2}.

Identification of G-Proteins by Hybridization Experiments. Since the immunoblotting studies did not provide conclusive evidence regarding the identities of the G-proteins in the olfactory epithelium, an alternative strategy was pursued. This strategy is based on the assumption that nucleotide probes may be more specific than polyclonal antisera that recognized only limited domains within the antigen to which they were raised, namely the peptide sequence used as antigen. Thus far, we have concentrated our efforts on identification at the mRNA level of the 45 kDa cholera toxin substrate which may represent the

catfish homolog of G_{olf}. Plasmids encoding rat G_{olf} and OMP were obtained from Dr. R. Reed in small (2 ug) quantities. To obtain sufficient material for the hybridization experiments, DH5 competent cells were transformed with each plasmid and grown in large-scale cultures. From 1 ng of plasmid, we routinely obtain about 250 ug of DNA. Our initial efforts have been directed to the preparation of labeled RNA probes from these plasmids. To this end, a nonradioactive labeling procedure has been employed to reduce exposure to radioactivity and to minimize radioactive waste. After linearization of the plasmid encoding G_{olf} with Pst I, in vitro transcription with T3 RNA polymerase in the presence of digoxigenin-11-dUTP yielded a 0.75 kb transcript. This transcript was significantly smaller than that obtained by T7 RNA polymerase-mediated transcription (Fig. 4). However, the probe obtained with T3 RNA polymerase did produce a weak signal when hybridized to mRNA isolated from catfish olfactory epithelium. The integrity of the isolated mRNA was evaluated by agarose gel electrophoresis (Fig. 4). A full-length RNA probe for G_{olf} has now been prepared by linearizing the vector with Not I. This probe will be tested in future experiments to identify the cholera toxin substrates in the olfactory epithelium. Labeled oligonucleotide probes to actin and tubulin have also been prepared by DNA tailing. These probes will be used to further evaluate the integrity of the isolated mRNA preparations, particularly those derived from nonchemosensory control tissues. Thus far, we have obtained undegraded mRNA from rat and catfish

olfactory epithelium and from catfish brain, liver, spleen, heart, and skeletal muscle, as judged by agarose gel electrophoresis. Hybridization experiments now in progress will be used to identify the G-proteins that may be unique to the olfactory epithelium.

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Table I. Peptide Antisera to Stimulatory G-Protein Alpha Subunits. The amino acid sequence to which each of the three antisera were produced is shown in the single letter code. Amino acid sequences enclosed by the solid lines indicate sequence differences between G_s and G_{olf}. The numbers below the sequences indicate the amino acid positions in the alpha subunit of G_{olf}. Antiserum RR3 specifically recognizes rat G_{olf} (25). Antisera 584 and RM1 cross-react with both G_s and G_{olf} (32, 34).

Antiserum		Peptide Sequence
RR3	Golf	K T [A] E D Q [G V D] E K [E E] R S A
	Gs	K T [ ] E D Q [R N E] E K [A Q] R E A
		8 22
584	Golf	T P [D A] G E D P [K] V T R A K [F]
	Gs	T P [E P] G E D P [R] V T R A K [Y]
		325 339
RM1	Golf	R M H L [K] Q Y E L L
	Gs	R M H L [R] Q Y E L L

FIGURE LEGENDS

Fig. 1. Immunoblotting of olfactory cilia and membranes from deciliated olfactory epithelium with antibody RR3. Isolated cilia (C) and deciliated (D) membranes were prepared from rat and catfish olfactory epithelium and were separated by SDS-polyacrylamide gel electrophoresis, transferred to Westran and probed with affinity-purified antibody (1:1000 dilution). Samples from rat contained 25 ug protein and samples from catfish contained 100 ug protein.

Fig. 2. Immunoblotting of olfactory cilia and membranes from deciliated olfactory epithelium with antibody 584 and RM1. Samples from rat and catfish were processed as described in Fig. 1. Antibodies were used at 1:1000 dilution. A) Immunoblotting with antibody 584. B) Immunoblotting with antibody RM1.

Fig. 3. Immunoblotting of olfactory cilia and membranes from deciliated olfactory epithelium with antiserum to G_{i1} and G_{i2}. Samples from rat and catfish were processed as described in Fig. 1. The antibody was used at 1:1000 dilution.

Fig. 4. Agarose gel electrophoresis of isolated mRNA and RNA transcripts. RNA samples (2 ug) were denatured with glyoxal and separated in 1.2% agarose gels in the presence of ethidium bromide. A) RNA isolated from catfish olfactory epithelium and brain after oligo-dT cellulose selection. B) <u>In vitro</u> RNA transcripts of G_{olf} RNA labeled with digoxigenin-11-dUTP obtained with T3 and T7 RNA polymerases.

Fig. 1

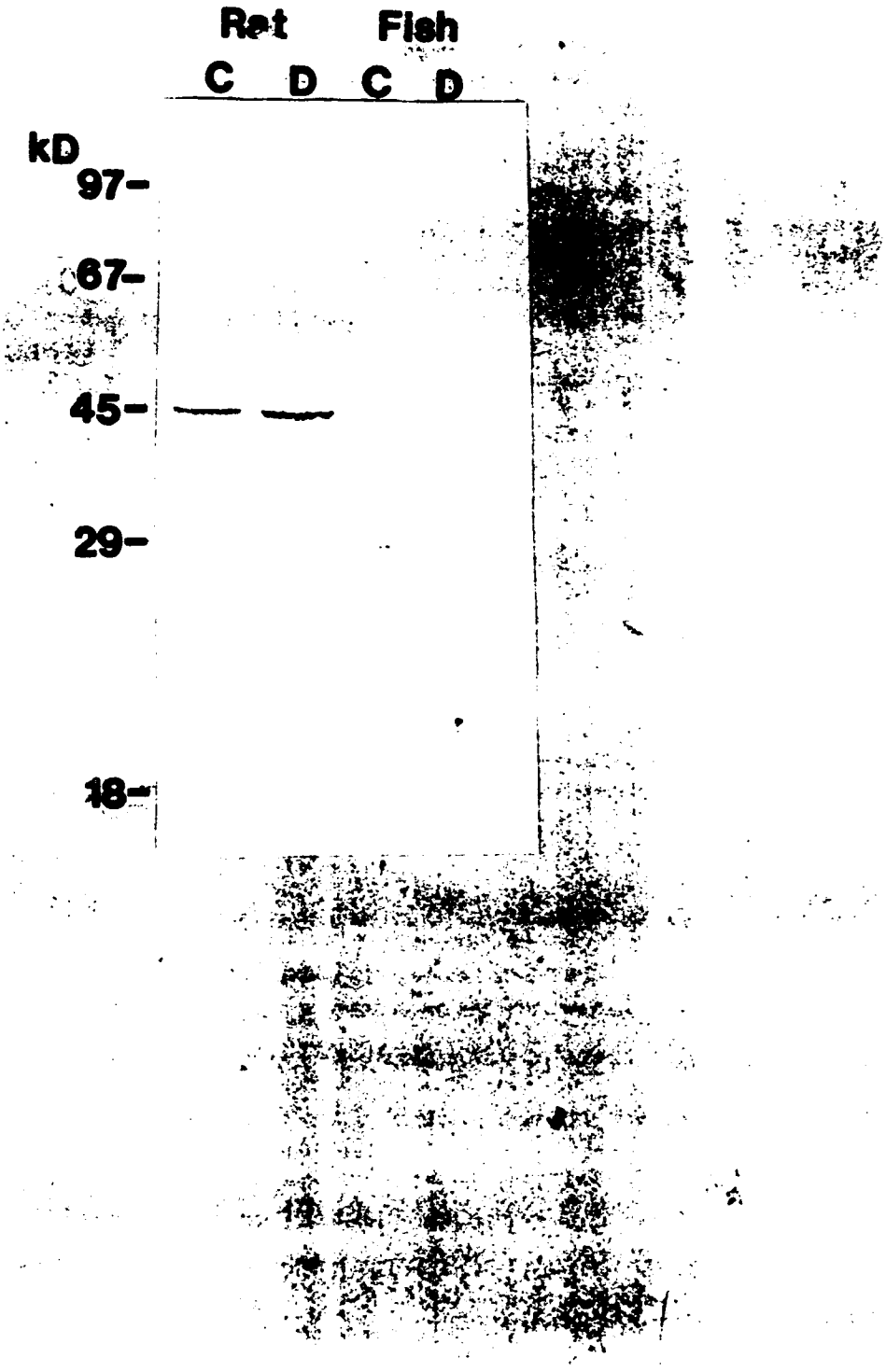


Fig. 2

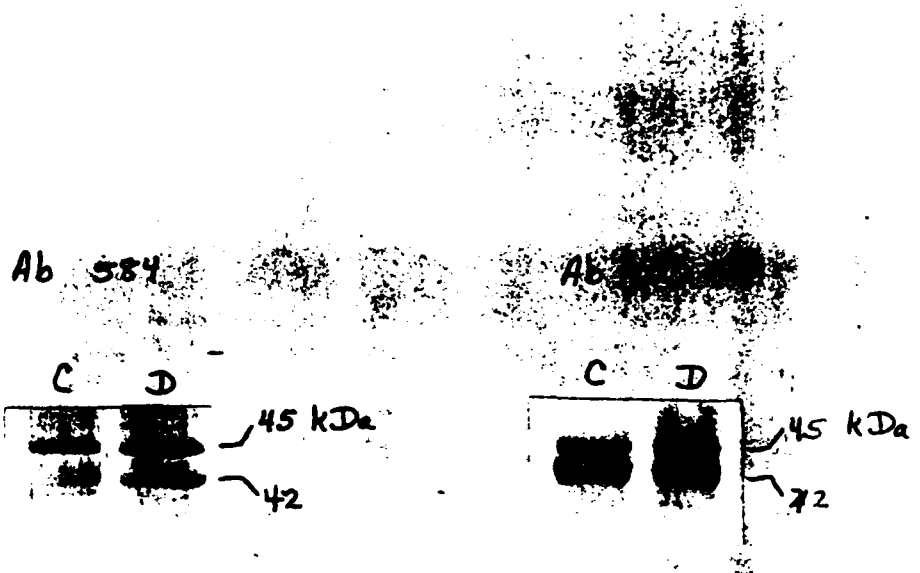


Fig. 3

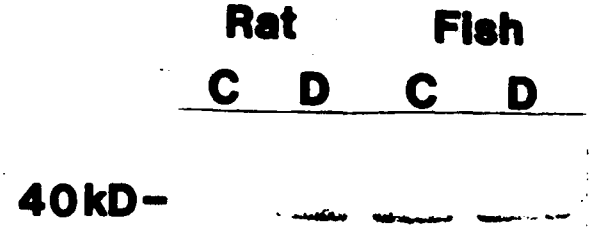
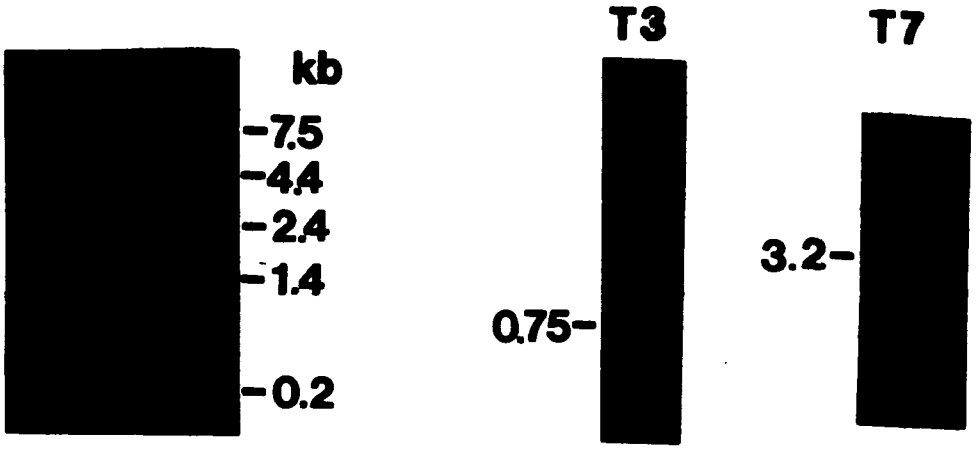


Fig. 4



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