

| |
|------|
| 新 制 |
| 理 |
| 661 |
| 京大附図 |

学位申請論文

上田 泰次

Monoclonal Antibodies to Chick Crystallins

Yasuji Ueda

Institute for Biophysics, Faculty of Science, Kyoto
University, Kyoto 606 Japan

Present Address: Department of Applied Biological Science,
Faculty of Science and Technology, Science University of
Tokyo, Noda 278, Japan

Key words: monoclonal antibodies; chick crystallins;
Immunoblotting.

Running Heads: Monoclonal Chick Antibodies Y. Ueda

Several monoclonal antibodies against chick crystallins were obtained. Immunoblot analysis indicated that the monoclonal antibodies distinguish not only the three classes of crystallin (α , β and δ), but also their subclasses. Monoclonal antibodies α -2 and α -1 reacted exclusively with α_A and α_B , respectively, demonstrating that α -crystallin subclasses of the chicken are antigenically distinct. An immunohistological study utilizing these monoclonal antibodies showed that the two α -crystallin subclasses, α_A and α_B , are co-expressed in the same cells and are more concentrated in the epithelium than the fibers in 14-day-old chick embryo lenses. However, immunofluorescence suggested different distribution of α -crystallin subclasses within an epithelial cell. α_A is distributed evenly in the cytoplasm, but α_B is more concentrated in the fiber-proximal side. Using anti- β monoclonal antibodies, it was shown that β -crystallins are divided into three distinct subclasses according to their antigenicity: 27-kDa and 25-kDa β -crystallins are recognized by β -1 antibody, 26-kDa and 19-kDa β -crystallins by β -2 antibody, and 35-kDa β -crystallins by β -3 antibody. All of the anti- δ -crystallin monoclonal antibodies (δ -1 to δ -3) obtained here bound to all δ -crystallin molecular species separable by isoelectrofocusing.

1. Introduction

Crystallins are the major lens-specific soluble proteins. Avian crystallins are composed of three classes, α -, β - and δ -crystallins, which are distinct in antigenicity. Production of monoclonal antibodies against chick crystallins is reported here for the following two major purposes.

Crystallins have been used as molecular markers to study lens differentiation. Localization of the crystallins in the lens and the timing of their appearance during lens development have been studied using conventional polyclonal antibodies (e.g. Clayton, 1970). However, very complex spatio-temporal patterns of crystallin expression require more specific probes for precise description. In addition, previous studies indicated the presence of δ -crystallin antigenicity in certain non-lenticular embryonic tissues such as adenohypophysis (Barabanov, 1977, 1982). Use of anti-crystallin monoclonal antibodies provides a straightforward approach to these various aspects.

There is cumulative evidence that crystallin classes are derived from gene families (Piatigorsky, 1984b). Interrelationships between the family members have been studied by comparing homologies in genomic and cDNA sequences (Hejetmancik and Piatigorsky, 1983; Piatigorsky, 1984a, b); however, no specific probes have been available for the crystallin polypeptide. The monoclonal antibodies described here not only provide such probes but, together with nucleic acid probes, will help to establish the relationships between the crystallin subclasses.

2. Materials and Methods

Production of hybridomas

Two 4-week-old Balb/c mice were immunized by i.p. injections with lens homogenates of 1-day-old chickens (five lenses/injection) and two others were immunized by injections of β -crystallin which were partly purified from lens extracts by gel filtration according to Ostrer and Piatigorsky (1980). Spleen cells from these animals and mouse myeloma cells (P3-X63-U1, Yelton, Diamond Kwan and Scharff, 1977) were fused with 50% polyethyleneglycol. Hybridomas were cultured in 96-well plates and selected in hypoxanthin aminopterin and thymidine (HAT) medium. First screening of hybridomas was carried out by solid phase antibodies binding assay (SABA). Multi-well plates were coated with purified α -, β - or δ -crystallin ($100\mu\text{g ml}^{-1}$) and bovine serum albumin (3%) in phosphate-buffered saline (PBS). The culture supernatant of each hybridoma well was added to crystallin-coated wells. After incubation at room temperature for 1 hr, the wells were washed with PBS, incubated with peroxidase-conjugated anti-mouse immunoglobulin antibodies (DAKO) for 1 hr, again washed with PBS and finally incubated with 0.1% ortho-phenylenediamine, 0.0015% hydroperoxide in 0.1 M sodium citrate buffer (pH 4.5) for 10 min. The hybridomas, the supernatants of which produced yellow to brown color in one of the crystallin wells, were transferred to a 24-well plate and cultured further. Secondary screening was carried out by immunoblot assay. Lens extracts of newly hatched chicks were electrophoresed (Laemmli, 1970) in SDS-polyacrylamide gel slab with sample application of full gel width, and transferred onto

nitrocellulose membrane electrophoretically according to Towbin Staehelin and Gordon (1979). The membranes were incubated in 4% bovine serum albumin in PBS at 4°C overnight, then cut vertically into several strips bearing the same set of protein bands. A strip was incubated with supernatant of a hybridoma, washed with PBS, incubated with peroxidase-conjugated anti-mouse Ig, washed, and finally incubated with 0.02% 3-amino-9-ethylcarbazole (made by 20-fold dilution of 0.4% solution in N,N'-dimethylformamide), and 0.03% hydroperoxide in 0.1M acetate buffer (pH 5.2). The hybridomas which produced antibody reactive to crystallin band(s) were cloned by a limiting dilution method. Cloned hybridomas were finally characterized by the SABA and immunoblot assay. The Ig class of each monoclonal antibody was tested by Ouchterlony method.

Immunoblot analysis

SDS-gel electrophoresis was carried out as described above. Isoelectrofocusing was performed in the horizontally cast 3.4% polyacrylamide gel containing 7.4% sucrose, 2.8% Ampholine (pH 5-7) and 0.56% Ampholine (pH 3.5-10) (LKB). Ten micrograms of soluble proteins from 1 day chick lens extracts in water were loaded on the gel, which had been pre-run at 50 mA for 30 min, and focused at 400 V for 30 min, 900 V for 90 min and 1200V for 3 min. After electrophoresis or focusing, proteins were transferred electrophoretically onto a nitrocellulose membrane. ¹²⁵I-anti-mouse Ig (Amersham) was used as secondary antibodies. After washing, the membranes were dried and exposed to RX-O mat films (Fuji), with intensifying screens, at -70°C overnight.

Immunohistology

Eyes of 14-day-old chick embryos were fixed with Carnoy's fixative and paraffin-sectioned. The sections were incubated with a monoclonal antibody, washed in PBS, incubated with fluorescein-conjugated rabbit Ig against mouse Ig (DAKO), and observed under a fluorescence microscope.

3. Results

Production of monoclonal antibodies

Two mice were immunized with chick lens homogenates and the spleen cells were fused with myelomas. From this first fusion, 384 hybridoma-containing wells were obtained, among which about half were positive for antibodies against chick crystallins as assayed by SABA. The majority of the positive wells were against δ , with 15 wells against α , and two wells against β . The hybridoma cells of a selected number of wells (all positives to α or β and 20 positive to δ) were transferred to 24-well plates, expanded and the activity in the culture supernatant was characterized by immunoblotting. The hybridomas of the wells unequivocally reactive to α -crystallin were cloned. Eventually, six monoclonal hybridoma lines (α -1, α -2, β -1, δ -1, δ -2 and δ -3) were established (Fig. 1).

To obtain a greater variety of anti- β -crystallin monoclonal antibodies, the second and third fusions were performed with mice immunized with partly purified β -crystallins. Among approximately 800 wells of hybridomas, 96 wells were reactive in SABA to β -crystallin. Of these 96, those which showed patterns of polypeptide reactivity different from that of β -1 in immunoblot were selected and hybridomas were cloned, and finally two hybridoma lines were established which produce anti- β -crystallin antibodies (β -2 and β -3) (Fig. 2).

Altogether, eight monoclonal antibodies: α -1, α -2, β -1, β -2, β -3, δ -1, δ -2 and δ -3, were obtained (Figs 1 and 2, and Table I).

Anti- α -crystallin antibodies: α -1 and α -2

These antibodies each recognize only one of the two α -crystallin subclasses. α -1 recognizes α_B (20 kDa) and α -2 recognizes α_A (18 kDa). There was no cross-reaction each other.

Anti- β -crystallin antibodies: β -1, β -2 and β -3

These antibodies recognize different subclasses of β -crystallins. β -1 recognizes 25-kDa and 27-kDa β -crystallin, β -2 recognizes 19-kDa and 26-kDa β -crystallins, and β -3 recognizes 35-kDa β -crystallin. Since seven different β -crystallin polypeptides were detected by SDS-gel electrophoresis (e.g. Hejtmacik and Piatigorsky, 1983), there seems to be at least one additional β -crystallin subclass not recognized by these antibodies. There was no detectable cross-reaction between the antibodies.

Anti- δ -crystallin antibodies: δ -1, δ -2 and δ -3

There is a microheterogeneity in δ -crystallin polypeptides; they are resolved into a number of bands by isoelectrofocusing (Brama and Starre, 1976; Piatigorsky, 1978). With our current isoelectrofocusing system, seven δ -crystallin bands are resolved (Fig. 3). The author examined whether anti- δ -crystallin antibodies distinguish subclasses of δ -crystallin polypeptides and by immunoblotting technique, I found that all bands were equally recognized by the monoclonal antibodies.

Immunohistology of crystallins using the monoclonal antibodies

Monoclonal antibodies obtained here were generally much less sensitive in immunohistological detection of the crystallins than

the conventional polyclonal antibodies; nevertheless, α -1, α -2 and δ -1 were reactive enough to immunohistological analysis at certain developmental stages of lens. The results of immunofluorescence staining of paraffin sections of chick eyes are shown in Fig. 4. As previously reported with polyclonal antibodies, δ -crystallins were found to be more enriched in lens fibers than in lens epithelium, and conversely α -crystallins are more enriched in the lens epithelium. α_A and α_B were co-expressed in the same and all epithelial cells. However, they appeared to be distributed differently within an epithelial cell. Immunofluorescence of α_A was distributed evenly in the cytoplasm, but that of α_B was higher in the fiber-proximal side (Fig. 4).

4. Discussion

α -crystallins

α -crystallins are composed of two subclasses, α_A and α_B , encoded by distinct genes (for review see Piatigorsky, 1984b), but have been regarded as a single antigenic class (e.g. Ikeda and Zwaan, 1967; de Pomerai, Prichard and Clayton, 1977). Amino acid sequence comparison indicated only 60% homology between α_A and α_B bovine crystallins (van der Ouderaa, de Jong, Hilderink and Bloemendal, 1974; de Jong, 1981). Nucleic acid data showed that α_A cDNA and genomic sequences do not hybridize with α_B sequences even under a low-stringency condition (Okazaki, 1986) indicating that they are not highly homologous. A previous study indicated that human α_A and α_B distinct and common antigenic determinants by using monoclonal antibodies (Church, Alongi and Church, 1983). In the present study, monoclonal antibodies specifically reactive to each α -crystallin subclass of the chicken were established. This clearly demonstrates that chick α_A - and α_B -crystallins have distinct epitopes.

Immunohistological analysis showed that α_A - and α_B -crystallins are co-expressed, more highly in the lens epithelium than in the fibers (Fig. 4). The present study also indicated different distributions of α -crystallin subclasses in the epithelial cells. The α -crystallins have been considered to form heterotypic aggregates between the subclasses (Siezen, Bindels and Hoenders, 1979). The composition of α -crystallin subclasses may be different between the aggregates, depending on the localization within a cytoplasm.

β -crystallins

At least seven different β -crystallin polypeptide species have been recognized by their molecular weights (Osterer, Beebe and Piatigorsky, 1980, Piatigorsky, 1984b). Previous immunological studies using polyclonal antibodies indicated that antigenic determinants of β -crystallins are highly complex (Clayton and Truman, 1974; de Pomerai et al., 1977). Though the study with monoclonal antibody to β -crystallin polypeptide has partly defined the β -crystallin antigenic determinants (Carper, Smith-Gill and Kinoshita, 1984), epitope compositions among β -crystallin polypeptides have not been clearly established.

In vitro translation analysis of mRNAs selected by hybridization to cloned cDNA sequences revealed six distinct homology groups: 26-kDa and 19-kDa β -crystallin are highly homologous and probably encoded by a single gene (Hejtmancik and Piatigorsky, 1983). In addition, it was found that mRNA of 27-kDa β -crystallin weakly hybridizes to 25-kDa β -crystallin cDNA. Interestingly, the same relationships were observed with the monoclonal antibodies. β -2 antibodies bind 19-kDa and 26-kDa β -crystallin with the same efficiency. On the other hand, β -1 antibody binds strongly to 25-kDa β -crystallin and weakly to 27-kDa β -crystallin. β -3 antibody binds distinct 35-kDa β -crystallin. Thus it is shown that β -crystallins are classified into simple antigenic subgroups in a way consistent with cDNA homology groups.

The expression of each β -crystallin subclass is subject to a complicate spatio-temporal regulation (Ostrer et al., 1980; Hejtmancik, Beebe, Ostrer and Piatigorsky, 1984). The monoclonal

antibodies are obviously useful in order to study the β -crystallin expression pattern because of the high selectivity between β -crystallin subclasses. It is unfortunate that anti- β -crystallin antibodies reported here do not have a high enough affinity to bind to crystallin in histological sections of fixed lenses, presumably due to the use of native β -crystallins as immunogen. The use of frozen sections may circumvent the problem. It may also be feasible to establish highly specific anti- β -crystallin monoclonal antibodies that are useful in histological analysis.

δ -crystallins

There are two non-allelic δ -crystallin genes found in the chicken. Evidence indicates that one of them, gene 1, is the active entity (Piatigorsky, 1984a). Isoelectrofocusing resolves the microheterogeneity of δ -crystallin polypeptides. In the present study it was demonstrated that all δ -crystallin bands demonstrated are equally recognized by three different monoclonal antibodies. Thus, although the origin of the heterogeneity is not known, it is clear that all δ -crystallins share some antigenic determinants.

Application to non-lenticular crystallin expression

Indications of the expression of crystallins in certain non-lenticular tissues of avian embryos have been observed (for review see Okada, 1983). These observations were made with techniques using polyclonal antibodies, and authenticity of the reactions was less certain than with monoclonal antibodies. There

is a report indicating immunological cross-reaction of feather keratins with δ -crystallin by using polyclonal antibodies (Kodama and Eguchi, 1983). Obviously, the monoclonal antibodies established here provide most clear-cut means of verifying these observations. Indeed, by utilizing the monoclonal antibodies, the author obtained definite evidence of δ -crystallin expression in embryonic adenohypophysis of the chicken (Ueda and Okada, 1986) and for expression of all crystallin classes in putative lens cells differentiated from pinealocytes (Watanabe et al., 1985).

ACKNOWLEDGMENT

I am most indebted to Professor T.S. Okada for an introduction to this study and for support. I thank Dr M. Takeichi for instruction on hybridoma techniques, and Dr M. Hino and Mr H. Michishita for technical assistance. I also thank Drs H. Kondoh, K. Yasuda and M. Takeichi for reading the manuscript.

REFERENCES

- Barabanov, V. M. (1977). Expression of δ -crystallin in the adenohipophysis of chick embryos. Dokl. Akad. Nauk. SSSR 234, 195-8.
- Barabanov, V. M. (1982). Localization of δ -crystallin in non-lens tissues of quail embryos. Dokl. Akad. Nauk. SSSR 262, 1491-4.
- Brama, S. K. and Starre H. V. D. (1976). Studies of biosynthesis of soluble lens crystallin antigens in the chick by isoelectrofocusing in thin-layer polyacrylamide gel. Exp. Cell Res. 97, 175-83.
- Carper, D., Smith-Gill, S. J. and Kinoshita, J. H. (1986). Immunocytochemical localization of the 27K β -crystallin polypeptide in the mouse lens during development using a specific monoclonal antibody: implications for cataract formation in the Philly mouse. Dev. Biol. 113, 104-9.
- Church, M. J., Alongi, A. and Church, R. L. (1983). Production and characterization of monoclonal antibodies to human alpha crystallin. Invest. Ophthalmol. Vis. Sci. ARVO Suppl. 157.
- Clayton, R. M. (1970). Problems of Differentiation in the vertebrate lens. Curr. Top. Dev. Biol. 5, 115-80.
- Clayton, R. M. and Truman, D. E. S. (1974). The antigenic structure of chick β -crystallin subunits. Exp. Eye Res. 18, 195-206.
- de Jong, W. W. (1981). Evolution of lens crystallins. In "Molecular and cellular biology of the eye lens." (ed. Bloemendal, H.) Pp. 221-78. John and Wiley; New York.
- de Pomerai, D. I., Prichard, D. J. and Clayton, R. M. (1977).

- Biochemical and immunological Studies of lentoid formation in cultures of embryonic chicken neural retina and day-old chick lens epithelium. Dev. Biol. 60, 416-27.
- Hejetmancik, J. F., Beebe, D. C., Ostrer, H. and Piatigorsky, J. (1984). α - and β -crystallin mRNAs levels in the embryonic and posthatched chicken lens: Temporal and spatial changes during development. Dev. Biol. 109, 72-81.
- Hejetmancik J. F. and Piatigorsky, J. (1983). Diversity of β -crystallin mRNAs of the chicken lens. J. Biol. Chem. 258, 3382-7.
- Ikeda, A. and Zwaan, J. (1967). The changing cellular localization of α -crystallin in the lens of the chicken embryo, studied by immunofluorescence. Dev. Biol. 15, 348-67.
- Kodama, R. and Eguchi, G. (1983). Characterization of an antiserum against feather keratins of the chick: Its crossreaction with a lens protein, δ -crystallin. Dev. Growth Differ. 25, 261-70.
- Laemimli, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 227, 680-5.
- Ohno M., Sakamoto, H., Yasuda, K., Okada, T. S. and Shimura, Y. (1985). Nucleotide sequence of a chicken δ -crystallin gene. Nucl. Acids Res. 13, 1593-606.
- Okada, T. S. (1983). Recent progress in studies of transdifferentiation of eye tissue in vitro. Cell Differ. 13, 177-83.
- Okazaki, K. (1986). Ph. D. Thesis Kyoto University.

- Ostrer, H. and Piatigorsky, J. (1980). β -Crystallins of the adult chicken lens. relatedness of the polypeptides and their aggregates. Exp. Eye Res. 30, 679-89.
- Ostrer, H., Beebe, D. C. and Piatigorsky, J. (1981). β -crystallin mRNAs: differential distribution in the developing chicken lens. Dev. Biol. 86, 403-8.
- Piatigorsky, J. (1978). Difference in isoelectric points and subunit composition of δ -crystallins synthesized in lenses cultured with and without vitous bodies. Exp. Eye Res. 27, 227-37.
- Piatigorsky, J. (1984a). δ -crystallins and their nucleic acids. Mol. Cell. Biochem. 59, 33-56.
- Piatigorsky, J.(1984b). Lens crystallins and their gene families. Cell 38, 620-1.
- Siezen, R. J., Bindels, J. G. and Hoenders, H. J. (1979). The relationship between monomeric, oligomeric and polymeric α -crystallin in the calf lens nucleus. Exp. Eye Res. 28, 551-67.
- Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: procedure and some applications. Pro. Nat. Acad. Sci. U.S.A. 76, 4350-4.
- Ueda, Y. and Okada, T. S. (1986). Transient expression of a 'lens-specific' gene, δ -crystallin, in the embryonic chicken adenohipophysis. Cell Differ. 16, 241-50.
- van der Ouderaa, F. J., de Jong, W. W., Hilderink, A. and Bloemendal, H. (1974). The amino-acid sequence of the α_{B2} chain of bovine α -crystallin. Eur. J. Biochem. 49, 157-68.

Watanabe, K., Aoyama, H., Tamamaki, N., Yasujima, M., Nojyo, Y., Ueda, Y. and Okada, T. S. (1985). Oculopotency of embryonic quail pineals as revealed by cell culture studies. Cell Differ. 16, 241-50.

Yelton, D. E., Diamond, B. A., Kwan, S. P. and Scharff, M. P. (1977). Fusion of mouse myeloma and spleen cells. Curr. Top. Microbiol. Immunol. 81, 1-7.

Figure Legends

Fig. 1. Autoradiograms of immunoblots, which show specificity of monoclonal antibodies. Soluble proteins from day-old chick lenses were separated in SDS-polyacrylamide gel (15%) and transferred onto a nitrocellulose filter. The filter was cut into strips, in some cases in the middle of the lane to examine the identity/non-identity of the recognized polypeptides. Strip 1 was incubated with β -1, strip 2 was incubated with α -2, strip 3 was incubated with α -1 and strip 4 was incubated with δ -1. CB indicates Coomassie brilliant blue-stained gel. Positions of δ -crystallin (50-kDa), β -crystallin (27- and 25-kDa), α_B - (20-kDa) and α_A - (18-kDa) crystallins recognized by one of the antibodies are indicated by arrows.

Fig. 2. Autoradiograms of an immunoblot using anti- β -crystallin monoclonal antibodies. After electrophoresis of day old chick lens and transfer onto a filter, the filter was cut into strips and each strip was incubated with monoclonal antibody as primary antibody. Strip 1 incubated with β -2; strip 2 incubated with β -1; strip 3 incubated with mixture of β -1 and β -2; strip 4 incubated with β -3. CB indicates Coomassie brilliant blue-stained gel.

Fig. 3. Autoradiograms of an immunoblot using anti- δ -crystallin monoclonal antibodies. After isoelectro-

focusing of day old chick lenses and transfer onto a filter, the filter was cut into strips each of which contains two lanes of bands of polypeptide. Each strip was incubated with anti- δ -crystallin monoclonal antibody as primary antibody. Strip 1, incubated with δ -1; strip 2 incubated with δ -2; strip 3 incubated with δ -3. CB indicates a gel stained with Coomassie brilliant blue after isoelectrofocusing of δ -crystallin.

Fig. 4. Indirect immunofluorescent staining of sections of chick lens with monoclonal antibodies to α -crystallin. (A) A section of 14-day-old chick embryo lens stained with α -1. (B) A nearby section of the same lens as (A) stained with α -2. (C) A control section of 14-day-old chick embryo lens stained with normal mouse serum. The bar indicates 100 μ m.

Table I. List of monoclonal antibodies against crystallins.

| Monoclonal antibody | α -1 | α -2 | β -1 | β -2 | β -3 | δ -1 | δ -2 | δ -3 |
|---|-------------|-------------|------------|------------|------------|-------------|-------------|-------------|
| Class of recognized crystallin | α_B | α_A | β | β | β | δ | δ | δ |
| Molecular size of antigen monomer (kDa) | 20 | 18 | 27,25 | 26,19 | 35 | 50* | 50 | 50 |
| Ig class | Ig M | Ig M | Ig M | NT** | NT | Ig G | Ig G | Ig G |

* Molecular weight of the δ -crystallins is calibrated as 50 kDa from an amino acid sequence, as deduced from cDNA and genomic nucleotide sequences (Ohno, Sakamoto, Yasuda, Okada and Shimura 1985).

** Not tested.

Fig 1

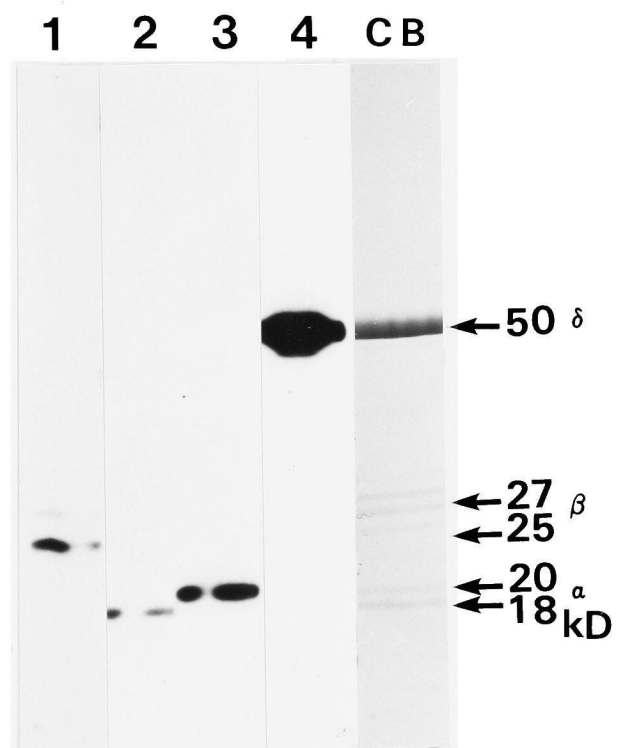


Fig 2

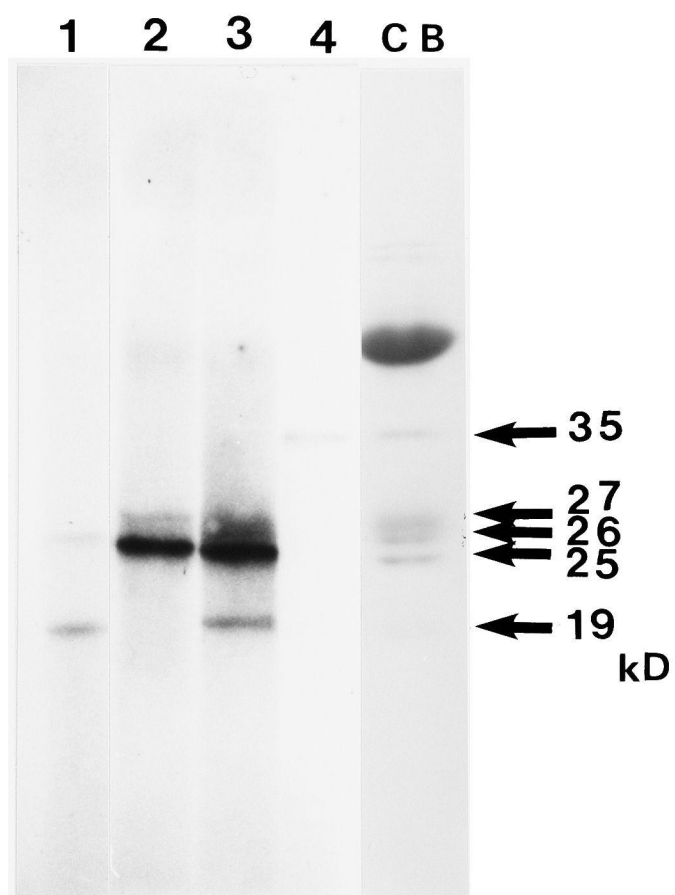


Fig 3

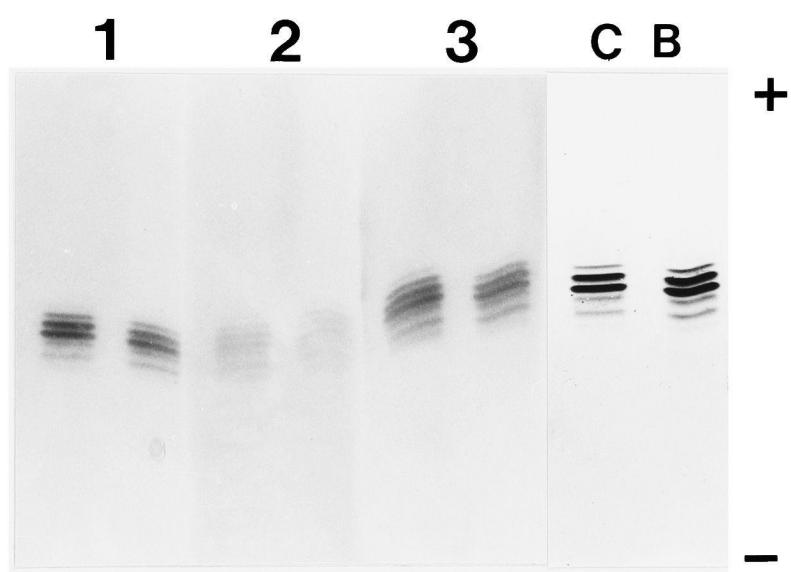


Fig 4

