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Production of Egg Yolk Antibody (IgY) Against Human Placental DNA-Dependent RNA Polymerase II

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Abstract: Polyclonal antibodies against human DNA-dependent RNA polymerase II (HPP II) were generated from chicken egg yolk after immunization with RNA polymerase II as an antigen. The antibodies from egg yolk (IgY) were purified and characterized. IgY showed a specificity against DNA-dependent RNA polymerase II, and was a polyclonal antibody against 12 subunits of polymerase II. An amount of 0.35 mg of IgY was obtained freman HPP II-Sepharose affinity column using 10 eggs from a chicken immunized against RNA polymerase II as an antigen. These antibodies can be used for isolating the genes for RNA polymerase II components, and for *in vitro* transcription assays using HP-RNA polymerase II.
Key words: Egg yolk Antibody (IgY), DNA-Dependent RNA polymerase-II (DNA-RNAPII), polyclonal antibody, IgY-purifications.

The animals most commonly used for the production of immune sera for diagnostic purposes are rabbits and guinea pigs, although horses, goats, and sheep are used to a lesser extent for the same purpose. Repeated attempts to obtain antibodies in either rabbits or guinea pigs to RNA polymerase II have been unseccessful (Stollar and Ward, 1970). Polymerase II purified to apparent homogeneity from both calf thymus and rat liver, and an RNA-polymerase-DNA complex, were used as antigen in these initial studies.

Ingles *et al.* (1975) obtained a potent antiserum after administration of calf thymus RNA polymerase II to hens. In the case of *Drosophila melanogaster* and *Chironomous thummi*, antisera against native RNA polymerase II were raised in mammals. Sheep antibodies to *Drosophila* RNA polymerase II were found to react with most polypeptides of insect RNA polymerase. They also reacted with the high molecular weight subunits of calf thymus RNA polymerase II (Kramer and Bautz, 1981). The response to antigenic stimuli is highly developed in fowl on both the cellular and humoral levels (Gottstein, 1981). In common with the colostra of mammals, bird egg yolks provide a supply of antibodies to chicks which, after absorption, confer passive immunity.

Carroll (1983) showed that the sera of hens immunized with killed *E. coli* bacteria produce circulatory immunogloublin which is 2-mercapto-ethanol sensitive and, therefore, taken to be an IgM type of antibody. No 2-mercapto-ethanol sensitive antibody has been found in the egg yolks of hens. From these experiments Malkinson (1965) concluded that IgM antibodies were not transferred to the yolks and that the antibodies in the yolks were of IgG types. Bar-Joseph *et al.* (1980) examined the transfer of antibodies to bovine serum albumin (BSA) from the circulation of hens to the yolk and, after correcting for the volume of solids in the yolk, concluded that the antibody levels of the yolk are the same or higher than the levels in the sera. Thus, hen blood serum IgG is transferred to the egg yolk to give acquired immunity to the offspring. The antibody in egg yolk has been called IgY (yolk immunoglobulin), because its proteins nature is somewhat different from mammalian IgG in molecular weight, isoelectric point, and binding with a complement (Gardner *et al.*, 1982). Also, a specific IgY against a given antigen (proteins, bacteria, and viruses) is produced in eggs from hens immunized with the antigen (Shimizu *et al.*, 1988).

For these reasons it was decided to use hens to produce antibodies against human placental RNA polymerase II which correctly transcribes *in vitro*. The prepara-

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tion of human placental RNA polymerase II contained some of the polymerase gene products and factors for transcription.

Using a polyclonal antibody preparation, polymerase II subunits and factors were also purified. These antibodies can be used as probes for isolating the genes for RNA polymerase II components, and possible factors for transcription.

Materials and Methods

Immunization of hens

Laying hens were obtained from a local egg farm and kept on regular light cycles. Hens were first injected subcutaneously at multiple sites with 100 μg of RNA polymerase II in complete Freund's adjuvant and with 50 μg of enzyme on days 14 and 21. Booster injections were given approximately once a month following the initial series with 50 μg of enzyme in incomplete adjuvant.

Antibody collection

Eggs were collected daily, marked for identification, and when suitable numbers were obtained IgY was extracted according to the method of Polson *et al.* (1980). Individual yolks were separated from the albumin (white) and thoroughly washed in a jet of water to remove as much of the albumin as possible. Intact yolks were dropped into a large glass funnel supported on a measuring cylinder. The fall into the funnel caused the yolk sacs to break and release the yolk which collected in the cylinder. The volume of yolk was measured and a volume of buffer equivalent to two volumes of yolk was added and thoroughly mixed with the yolk. Polyethylene glycol (PEG) which had been pulverized was added to a final concentration of 3.5% by weight of polymer to a volume of diluted yolk. The mixture was stirred until all the polymer was dissolved. The mixture was centrifuged in a Sorvall centrifuge at 14,000 x g for 10 min. This operation caused a separation into three phases in the centrifuge tubes. These were, ① a yellow fatty layer on the surface of the fluid, ② a clear supernatant layer (SNF), and ③ a semi-solid pliable layer of casein-like vitellin representing about 1/3 of the total volume in the centrifuge tubes. The SNF with the fatty layer was carefully decanted into a funnel with filter paper. This filter paper filtered off lipids and separated them from SNF. The volume of the clear filtrate was measured and more pulverized PEG was added by gentle stirring to adjust the final polymer concentration to 12 g PEG in 100 ml of SNF. At this concentration the PEG caused complete displacement of the IgY. A certain percentage of associated proteins, notably A and B livitin, co-precipitated with the IgY. These precipitated proteins were centrifuged off at 14,000 x g for 10 min in a Sorvall centrifuge. The pellet was redissolved in the original volume of yolk in a phosphate buffer, and the IgY was once again precipitated with 12% pulverized PEG, then centrifuged.

The pellets obtained were compacted by subjecting them to a centrifugal force of 14,000 x g for 10 min, and the exuded solution of PEG entrapped in the pellet was removed. In this manner the polymer which contaminated the IgY was reduced to a level at which it would not interfere with the antigen-antibody reaction at a high concentration of the antibody.

The final pellets were dissolved in a volume of buffer equivalent to half the volume of yolk from which they were extracted. The NaN contained in the buffer was adequate to preserve the IgY. For the complete removal of traces of PEG, IgY can be precipitated with 2 M $(NH_4)_2SO_4$ followed by centrifugation. In this instance PEG will form a liquid phase on the $(NH_4)_2SO_4$ solution. PEG may also be removed by precipitation of the IgY with 40% ethanol at a sub-zero temperative: the PEG will be soluble in the ethanol. In this study 40% ethanol at a sub-zero temperature was used for precipitation of IgY.

DEAE-cellulose purification of IgY

IgY was further purified by DEAE-cellulose chromatography by adsorption at 0.015 M K_2PO_4, pH 8.0, and elution with a 0.015 M to 0.30 M K_2PO_4 (pH 8.0) gradient.

Affinity column purification of IgY

Up to 20 ml of IgY (5 mg/ml) was applied to a column of purified RNA polymerase II conjugated to CNBr-activated Sepharose 4B (1 mg of enzyme/5 ml of gel). The column was washed to a low optical density with PBS, BBS-Tween (0.1 M boric acid, 0.025 M sodium borate, 1 M NaCl, pH 8.3) and PBS successively, then eluted with 0.5 acetic acid pH 3.0. The eluted antibody was neutralized and dialyzed against several changes of PBS.

Results

In order to isolate antibodies against human placental RNA polymerase II, the method of Polson *et al.* (1980) for isolation of antibodies from egg yolks of immunized has was used. Fifty μg polymerase II preparations (Fig. 1) were injected into three chickens every other week. After two months, IgY was isolated by the PEG-extraction method in order to check for IgY production. Ouchertolony tests were performed. After separating the

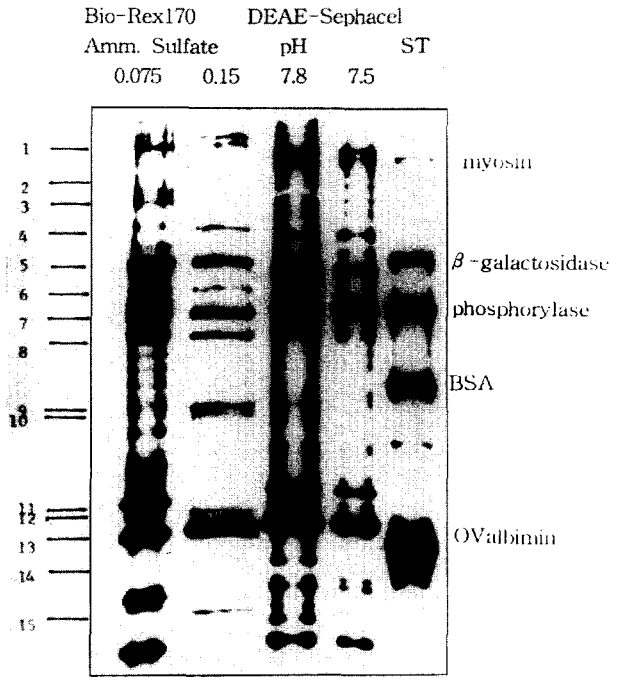

Fig. 1. SDS-polyacrylamide gel electrophoresis of RNA polymerase II after BioRex-70 column elution. Human placental RNA polymerase II, after 0.33 M ammonium sulfate elution on DEAE-Sephadex column was loaded onto a Bio-Rex 70 column (1 ml). Concentrations of 0.075 M and 0.15 M ammonium sulfate in TEDG were used for elution, and 0.14 M, 0.30 M and 0.40 M NaCl in triethanolamine buffer were also used for elution. I. Ammonium sulfate elution 0.33 M, on a DEAE-Sephadex column. Values of pH 7.5 and pH 7.8 TEDG were used for polymerase preparations. II. Bio-170 column elution of RNA polymerase II. 8% Laemmli SDS-PAGE was used for polypeptide separations.

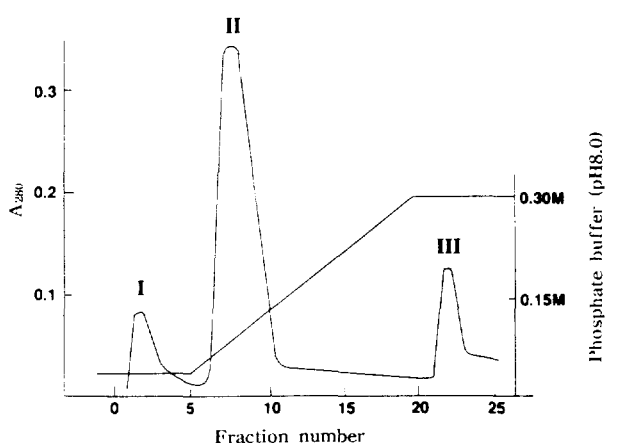

Fig. 2. Elution of polyclonal antibodies (PEG preparation) on a DEAE-Sephadex column. IgY from 10 eggs was purified by PEG purification methods, then loaded on a 100 ml DEAE-Sephadex column. Peak II, which was purified anti-IgY, was eluted with a 0.01 M (pH 8.0) to 0.3 M (pH 8.0) phosphate buffer linear gradient. The presence of anti-IgY in each peak was confirmed by an Ouchterlony test.

yolk and albumin layers, 3.5% polyethylene glycol was added to the yolk in order to separate the lipids and

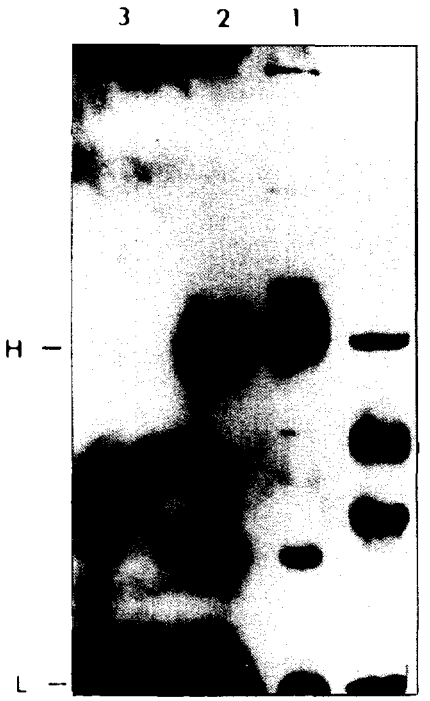

Fig. 3. Sodium dodecyl sulfate-gel electrophoresis of antibodies extracted from egg yolks. Five μg of protein was applied to 6%/8 % Laemmli SDS-polyacylamide gel and visualized with silver staining. Lane 1 : IgY purified through polyethylene glycol precipitation; Lane 2 : IgY purified by DEAE-cellulose chromatography. H and L denote the position of the heavy and light immuno-globulin chains.

vitellin. The immunoglobulin IgY from the vitellin layer was isolated by precipitating IgY by adding 12% PEG. The IgY extracted in this procedure was obtained in the amount of approximately 1 mg per egg from non-immunized animals, and up to 3 mg per egg from hyper-immunized hens. As shwon in Fig. 4, and Ouchterlony test after PEG preparation showed strong immunoprecipition bands.

In order to check whether PEG-purified IgY was homogeneous, 5 μg of PEG-purified IgY was run on 8% Laemmli SDS-PAGE. In addition to two distinctive bands of heavy and light chains of immunoglobulin, several contaminant polypeptides appeared (Fig. 3, lane 1). The PEG-purified antibody was further purified by DEAE-cellulose chromatography. PEG-purified IgY from 10 eggs (30 mg) was applied to a 100 ml DEAE-Sephadex column. After loading, the column was washed with 30 ml 0.015 M K_2PO_4, pH 8.0 and the IgY was eluted using a 100 ml 0.015~0.3 M K_2PO_4, gradient. As shown in Fig. 2, three peaks appeared during elution. IgY was confirmed by an Ouchterlony test. Peak II, which was eluted using a 0.04~0.11 M phosphate buffer (pH 8.0) gradient, showed cross-reactivity during the Ouchterlony test (Fig. 4) while peaks I and III showed no cross-reactivity.

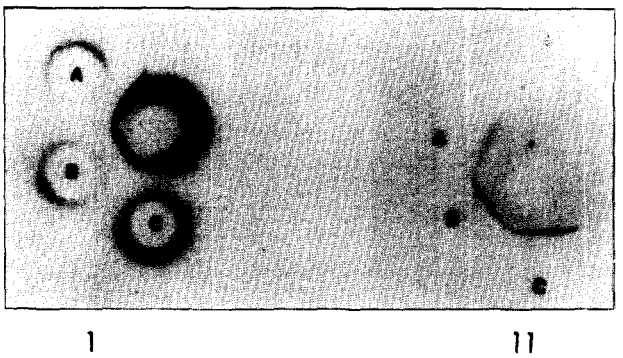

Fig. 4. Ouchterlony gel diffusion test of polymerase against polyethylene glycol precipitated IgY and DEAE-cellulose purified IgY. The center well contained PEG precipitated IgY (I) and DEAE-cellulose purified IgY (II). Wells A, B, and C contained 5 μg, 7 μg, and 10 μg of human placental RNA polymerase II, respectively. Peaks I and III in Fig. 18, which do not show any immunoprecipitation bands, were controls.

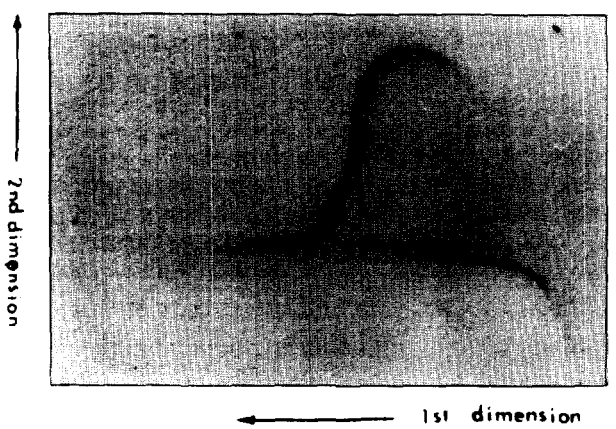

Fig. 5. Two dimensional Lawrell electrophoresis diagram of human placental RNA polymerase II against DEAE-cellulose purified anti-IgY. First dimension, 10 μg of polymerase was run electrophoretically on agarose. In the second dimension, separated polymerase was migrated into a layer of agarose that contained DEAE-cellulose purified IgY (1%).

In order to check the homogeneity of DEAE-cellulose peak II fraction purified IgY, 5 μg of IgY were run on Laemmli 8% SDS-PAGE. Distinctive bands of heavy and light chains of immunoglobulin were identified, though a few contaminant bands still appeared on the gel (Fig. 3, lane 2). In order to quickly check whether the DEAE-cellulose purified antibodies were complex antibodies against complex polypeptides, two dimensional Lawrell immunoelectrophoresis of human placental RNA polymerase II was done (Fig. 5).

In the first dimension, polymerase was run electrophoretically on agarose, then, in the second dimension separated polymerase was migrated in an electric field from a well into a layer of agarose containing antibidies. As shown in Fig. 5, immunoprecipitation bands in the agarose plate containing anti-RNAP II were formed. More than three bands on the agarose layer containing the antibody were seen. Antibodies purified in this experiment were antibodies against multi-polypeptides in polymerase II preparations. The antibodies purified on a DEAE-cellulose column might contain antibodies that are not specific to RNA polymerase II preparations.

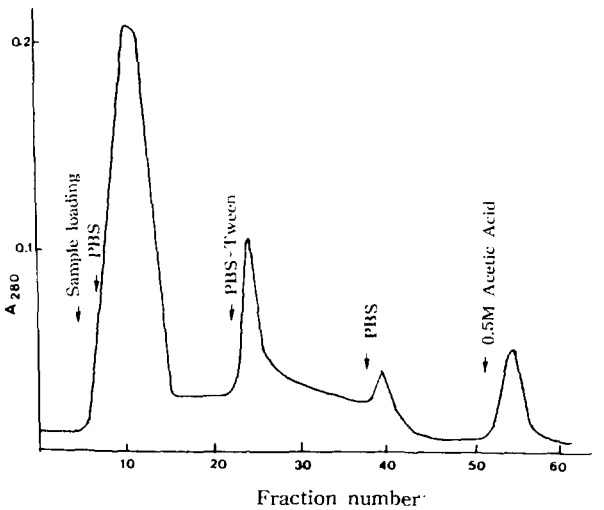

Fig. 6. Elution of polyclonal antibodies (DEAE-Sephadex preparation) from a RNA polymerase II-Sepharose 4B affinity column. DEAE-Sephadex purified IgY was further purified on an RNA polymerase II-Sepharose 4B affinity column. Up to 2 ml of IgY (5 mg/ml) was applied to a column of purified RNA polymerase II conjugated to CNBr-activated sepharose 4B. An amount 1.6 mg of DEAE-Sephadex purified IgY was loaded on a 1 ml affinity column. The column was washed to a low optical density (A= 280) with PBS, BBS-Tween, and PBS, successively, then eluted with 0.5 M acetic acid. The eluted antibody was neutralized and dialyzed against changes of PBS.

For the enrichment of anti-RNA polymerase II IgY, a column of purified RNA polymerase II conjugated to CNBr-activated Sepharose 4B was used. Coupling of polymerase II to CNBr-activated Sepharose4B was performed. An amount of 1.6 mg of DEAE-cellulose purified IgY was loaded onto a 1.0 ml affinity column. The column was washed with 20 ml of PBS to a low optical density with 10 ml of BBS-Tween (1 M saline, 0.1 M boric acid, 0.025 M Naborate, 0.1% Tween-20) and 5 ml of PBS, successively. IgY was eluted with 15 ml of 0.5 M acetic acid. The eluted antibody was neutralized and dialyzed against several changes of PBS. Fig. 6 shows the affinity column profile of IgY purification on the RNA PII-Sephadex 4B affinity column. IgY against polymerase II was eluted with 0.5 M acetic acid.

Table 1 shows the IgY purification steps from immunized chicken yolks. Five eggs were used for this preparation. Units were determined by ELISA. One unit was determined by the optical density value of IgY

Table 1.

Fraction number	Purification steps	Volume	Total protein	Unit	Specific activity	Purification fold
1	PEG	3.2 ml	7.68 mg	0.082	0.0106	–
2	DEAE-cellulose	1.8 ml	1.66 mg	0.046	0.283	44.0
3	HPP II-Sepharose column	0.7 ml	0.35 mg	4.366	12.47	1176.0

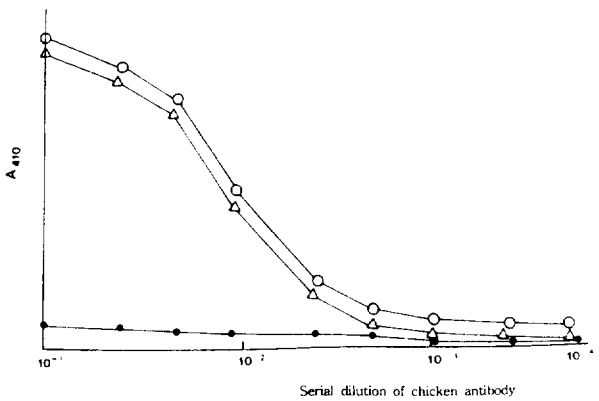

Fig. 7. Enzyme-linked binding immunoassay of chicken antibodies to RNA polymerase II. Varying dilutions of chicken antibodies in PBS were incubated in microtiter wells coated with RNA polymerase II (5 μg). The enzyme-linked immuno assay was done with black chiken IgY, green chicken IgY, and non-immune DEAE-eluted IgY.

after ELISA, when 10 μg of IgY was used from three different preparations. Ten μg/ml of HP polymerase II was used as an antigen for ELISA. As shown in Table 1, the antibody enriched by the affinity column had an approximately, 1,000-fold greater specific activity than PEG-purified IgY.

Discussion

Hens immunized with RNA polymerase II laid one egg per day for the duration of the immunization schedule. IgY was purified in three different steps, as shown in Table 1. IgY extracted by polyethylene glycol precipitation was obtained in amounts of approximately 1 mg per egg from nonimmunized animals, and up to 3 mg per egg from hyper-immunized hens. A shown in Fig. 3, lane 1, IgY purified by the PEG method was not homogeneous when analyzed by SDS-PAGE. The antibody was further purified on DEAE-cellulose chromatography (Fig. 2) by adsorption at 0.015 M K_2PO_4, pH 8.0 and elution with a 0.015~0.30 M K_2PO_4, pH 8.0 gradient. Peak II, which was eluted with a 0.04~0.11 M phosphate buffer (pH 8.0) gradient showed strong cross-reactivity on an Ouchterlony test (Fig. 4). Results showed that the IgY prepared by PEG, and DEAE-cellulose purified IgY both still showed some minor contaminant bands on SDS-PAGe (Fig. 3).

Fig. 8. Immunoblotting of RNA polymerase II subunits separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Lane a : RNA polymerase II was visualized with a silver stain after electrophoresis in 6%/8% SDS-PAGE; Lane B : The RNA polymerase II blot was treated with affinity purified IgY. I-labelled protein A, washed, dried, and autoradiographed; Lane c : The identical blot was treated with DEAE-cellulose purified IgY.

DEAE-cellulose and PEG IgY preparations are generally accepted procedures for preparing any kind of IgY, so a polymerase II-Sepharose affinity column was used to isolate specific anti-polymerase II IgY. The experimental results in Table 1 show that IgY isolated in this way had an approximately 1,000 times higher specific activity than PEG IgY preparations, and 27 times higher specific activity than DEAE-preparations. The protein amount after the affinity column, using five eggs, was approximately 350 μg.

ELISA is one of the most sensitive methods of detec-

ting cross-reactivity between an antigen and an antibody. Affinity-purified IgY was tested by ELISA for its cross-reactivity with HP polymerase II. As the results in Fig. 7 show, affinity-purified IgY (350 μg/ml) was serially diluted for ELISA. In this experiment 50 μl of IgY was used for an antibody reaction. A 10-fold dilution, which contained 0.155 μg of IgY. Fig. 7 shows that IgY purified from two different chicken eggs decreased in optical density as the IgY concentration decreased. Non-immune DEAE-purified IgY had no anti-polymerase II activity.

To determine which enzyme subunits are recognized by the specific IgY, the ability of antibodies to react with RNA polymerase II polypeptides separated by SDS-polyacrylamide gel and blotted onto nitrocellulose paper, was tested. Experimental results shown in Fig. 8 indicate reactivity with RNA polymerase II, including one DNA binding subunit (MW 195 kd). No anti-actin was found in this yolk IgY preparation. Both DEAE-purified IgY and affinity-purified IgY were used for immuno-blotting. Though they showed the same cross-reactivity with twelve different polypeptides, affinity-purified IgY showed much less non-specific binding on NC-paper.

This study indicates that specific antibodies to an RNA polymerase II preparation can be extracted in large quantities from the egg yolks of immunized hens. These results also indicate that the use of laying hens as hosts for immunization may be generally applicable to studies of other highly-conserved mammalian antigens.

These antibodies were present in high concentrations. They reacted with the native enzyme and removed it from solution in immune complexes with anti-chiken IgY, and they recognized twelve individual subunits of the enzyme.

These antibodies were used for a comparison of cross-reactivity with polymerases from other sources (data not shown). Antibodies against RNA polymerase II can be used for isolating the RNA polymerase II subunit genes, and can be a useful tool in verifying the real RNA polymerase II subunits during transcription reaction in the cell.

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