Molecular cloning of the chicken avidin cDNA

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ABSTRACT

A cDNA for chicken avidin was identified in a chicken oviduct cDNA library by screening with antibodies and synthetic oligodeoxyribonucleotides. Four recombinant clones were characterized and each contained the sequence of the oligonucleotide probes used in screening. They were capable also of expressing an antigen recognizable by a polyclonal or a mixture of monoclonal antibodies raised against avidin. The longest clone, λ cAV4, contained the entire coding sequence of avidin along with a signal peptide of 24 amino acids. An avidin mRNA, approximately 700 nucleotides in length, was induced by a single injection of progesterone over a period of twenty four hours. The avidin mRNA was distributed in a tissue-specific manner, since detectable concentration of the mRNA appeared only in the oviduct after stimulation with progesterone alone or with a combination of progesterone and estrogen. No avidin mRNA was detected in the liver or kidney under these conditions. Preliminary results on the genomic complexity of avidin suggest a single copy gene. Isolation of the natural gene for avidin and studies on its regulation now can be initiated using the cDNA probe.

INTRODUCTION

Avidin is widely distributed in avian, reptilian and amphibian species (1-6,9). It is considered a secretory protein, since it has been found to be synthesized in hen oviduct and concentrated in egg white (1,10). Synthesis of avidin is induced in the oviduct by a steroid hormone, progesterone (5,9,10). Induction of avidin has been used as a specific indicator of progesterone action in the oviduct mucosa as one of the first model systems to study the mechanism of regulation of a specific gene by steroid hormones (7-10). The regulation of avidin synthesis by progesterone has been proposed to occur mainly at the transcriptional level (8-12). A single injection of progesterone in vivo is sufficient for induction of avidin in the oviduct of diethylstilbestrol pre-treated chicks.

Avidin has been found also to be included in connection with tissue damage and inflammation caused by toxic doses of actinomycin D, septic E. coli or viral infections, or thermal injury in chicken (13-15). The

induction occurs within hours locally or systemically and it is suggested that besides being an oviductal progesterone-induced protein, avidin is also a progesterone-independent secretory product in areas of inflammation (14-16). The biological function of avidin in either case has not been elucidated yet although its bacteriostatic capacities have been suggested to be of selective advantage to vertebrates.

Avidin ($M_r = 66,000 - 69,000$) is a tetrameric basic glycoprotein constructed of four identical subunits ($M_r = 15,500 - 15,800$). The amino acid sequence of the peptide chain has been determined (17-20). The most characteristic property of the protein is its strong binding to the vitamin biotin ($K_d = 10^{-15}M$), with 4 moles of biotin per one mole of avidin (20-22). Avidin constitutes approximately 0.05% of hen oviduct total protein (23). We report here the isolation of the cDNA for the chicken avidin. Such a molecular probe should prove useful in studying the regulation by steroid hormones of the avidin gene in the avian reproductive tract, as well as the events occurring during inflammation and infections.

MATERIALS & METHODS

Materials

Immature female White Leghorn chicks were obtained from Texas Animal Specialties, Humble, Texas. All restriction and DNA modifying enzymes were purchased from Bethesda Research Laboratories, Pharmacia-PL Biochemicals, New England BioLabs or Boehringer Mannheim. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences (St. Petersburg, Fl).

Synthetic Oligonucleotide Probes

Two deoxyoligonucleotide probes (24-mer and 27-mer) carrying deoxyinosine residues (I) at positions corresponding to ambiguous nucleotides (24) derived from the known amino acid sequence of avidin were synthesized by the phosphotriester method with an Applied Biosystems 380A DNA synthesizer. The two oligonucleotides were combined and labelled with $\gamma\text{-}^{32}\text{P}\text{-ATP}$ and T4 polynucleotide kinase (25), and the unincorporated ATP was removed by chromatography on Sephadex G-25. The excluded fraction was added directly to the hybridization mixture.

Preparation of RNA

Tissue samples from hormone-treated chicks (as described in the figure legends) were rinsed in cold saline, immediately frozen in liquid nitrogen and stored at -70°C . Total RNA was isolated from the frozen tissue by a guanidine thiocyanate method (26). Poly(A)⁺-RNA was purified by two cycles

of chromatography on oligo(dT)-cellulose Type 3 (Collaborative Research) (27).
Construction and Screening of the Chick Oviduct cDNA Library

Total poly(A)⁺-RNA was isolated from the oviduct of laying hen. Double stranded cDNA was synthesized according to the method of Gbler and Hoffman (28) and used to generate a λ gt11 expression library containing 5.3×10^6 recombinant bacteriophage as described by Young and Davis (29,30).

Antibody probe: E. coli Y1090 cells were infected with the chick oviduct λ gt11 cDNA library and plated onto agar plates or onto plates containing 2 μ g/ml biotin and incubated at 42C for 3-4 hrs. The plates were overlaid with a dry nitrocellulose filter that had been presoaked in 10 mM IPTG and incubated at 37C for 3-4 hrs. The filters were incubated with an affinity purified polyclonal or a mixture of two specific monoclonal antibodies, α Av2 or α Av9, to chicken avidin (33). In the case of monoclonal antibodies, the filters were further incubated with an affinity purified rabbit anti-mouse IgG. The bound antibodies were detected with ¹²⁵I]-labeled protein A (34).

Oligonucleotide Probes: The temperature of hybridization for oligomers (T_H) was calculated by the following formula: $T_H = T_D - 5^\circ\text{C}$ where $T_D = 2^\circ\text{C} \times$ (the number of A-T base pairs) + $4^\circ\text{C} \times$ (the number of GC base pairs) (25,31). The substituted deoxyinosines are considered as "inert bases" (24) with little contribution to hybrid formation, or stability of the hybrid. Accordingly the calculated temperature of hybridization of the 24-mer oligonucleotide is about 44-5 = 39C and of the 27-mer is about 50-5 = 45C. Hybridization using a mixture of these two probes was carried out at an average temperature of 42C. The filters containing denatured bacteriophage DNA (32) were prehybridized in 6XSSC/5X Denhardt's/10mM EDTA, pH 7.5/100 μ g per ml heat denatured tRNA/0.5% SDS for 4 hrs at 42C. Hybridization was performed in 6XSSC/5X Denhardt's/0.1% SDS/250 μ g per ml heat denatured tRNA containing 10^7 cpm per ml of the oligonucleotide probe (specific activity of $1-4 \times 10^8$ cpm per μ g) at 42C for 18-20 h. Filters were washed 4 times with vigorous shaking for 5-10 min at room temperature in 6XSSC/0.5% SDS followed by a 1 min wash in the same solution at 42C. Additional clones were isolated by screening with ³²P-labeled cDNA inserts prepared from recombinant avidin clones. Hybridization conditions were as described by Maniatis et al. (32).

DNA Sequence Analysis

The cDNA inserts were digested with Eco RI and subcloned into the M13 phage vector system (35). Analysis of the DNA sequence was carried out by the dideoxynucleotide chain termination method (36).

DNA and RNA Blot Hybridization Analysis

Samples of total oviduct RNA (25 µg) were denatured with formamide and formaldehyde, separated by electrophoresis on a denaturing formaldehyde/agarose gel and transferred to a nitrocellulose filter as described in detail elsewhere (37). Genomic DNA from the chick spleen (10 µg/lane) was digested to completion with either Bam HI or Hind III, separated in an agarose gel, transferred to a nitrocellulose filter, and analyzed by the DNA blot hybridization method of Southern (38). RNA and DNA filters were hybridized to the ³²P-labeled insert of λcAV2 cut with Hind III to remove the poly A tail.

Dot-Blot Hybridization Analysis: Total RNA samples, isolated from the oviduct, liver or kidney following hormone administration, were dotted onto Biocytne A transfer membranes (Pall, 1.2 micron rating) by a manifold apparatus (Schleicher and Schuell) according to the protocol of Schloss *et al.* (39). Equal amounts of yeast RNA were included as controls for non-specific hybridization (not shown). The filters were hybridized with the ³²P-labeled insert of λcAV2 cut with Hind III to remove the poly (A) tail. Parallel filters were hybridized with a ³²P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA insert (40).

RESULTS AND DISCUSSION

Isolation of Chicken Avidin cDNAs

Altogether 700,000 recombinant clones from a chicken oviduct λgt11 cDNA expression library were screened at high density using the polyclonal antibody or a mixture of two synthetic oligonucleotides as a probe. Deoxyinosine was substituted into ambiguous third base positions of the amino acid codons. The oligonucleotide probes were ITC IAT IAA ICA ITG ICC IGT IAA (24-mer) and IAA ITT CCA ITT IAC IGT IAA ICC IAA (27-mer) which were complementary to the mRNA encoding amino acid residues 79-86 (24-mer) and 64-70 (27-mer). Two recombinant phages, λcAV1, (397 bp) and λcAV2 (384 bp) were isolated by screening with the polyclonal antibody and the synthetic oligonucleotides, respectively. The third cDNA clone, λcAV3, was detected by screening with monoclonal antibodies and by cross-hybridization to the oligonucleotide probes. Subsequent DNA sequence analysis of λcAV1 and λcAV2 showed that the inserts contained DNA encoding for the amino acid residues 39-128 of the chicken avidin (18) in addition to 85 nucleotides of 3' untranslated region and the beginning of a poly (A) tract (see following section). The sequence of the 250 bp insert from λcAV3 was found to be

Figure 2 Expression of the Avidin mRNA in the Chick Oviduct

Two week old, immature female White Leghorn chicks were treated for 12 days with diethylstilbestrol (DES) [2 mg/bird/day]. The birds were withdrawn from hormone treatment for 5 days, and then restimulated in groups of thirty with a single dose of either DES (2 mg/bird), progesterone (1 mg/bird), or both together. The animals were sacrificed at the times indicated and total cellular RNA isolated from the oviduct tissue as described. Total cellular RNA also was isolated from the oviduct of birds which were treated chronically with DES for 18 days. Twenty-five μ g of each RNA was electrophoresed on a formaldehyde agarose gel and blot-hybridized with the 32 P-labeled cDNA insert from λ CAV2. We estimate that the error in this gel electrophoresis system is approximately 12%.

DNA Sequence Analysis

The complete nucleotide sequence of avidin cDNA was determined and is shown in Fig. 1. The amino acid sequence deduced from the nucleotides 116-499 was identical with that determined for chicken avidin by DeLange and Huang (18), giving unambiguous confirmation of the cDNA clones. The sequences of both synthetic oligonucleotides used for screening were contained in all four cDNA inserts.

The longest clone, λ AV4, contained additional sequence further upstream beyond the coding region. Since avidin is a secreted protein, a signal peptide was expected at the N-terminal end of preavidin. The signal sequence of 24 amino acids preceding the first amino acid of the coding

Figure 3. Tissue Distribution and Steroid Hormone Regulation of Chick Avidin

Animals were hormonally treated for 24 hrs as described in Fig. 2. Total cellular RNA was isolated from the oviduct, liver and kidney; 10, 5, and 2.5 µg quantities were dotted onto Biodyne A transfer membranes.

Panel A: Hybridization with the 32 P-labeled cDNA insert from λ AV2.

Panel B: Hybridization with a 32 P-labeled cDNA for chick GAPDH.

sequence was revealed from sequence analysis of λ cAV4. It contains a Ser at position 113-115, one of the six commonly found amino acid residues in the cleavage site preceding the first coding amino acid of secretory proteins (43), as well as a characteristic highly hydrophobic central core containing seven Leu residues (nucleotides 60-85). In spite of the same length of the signal peptides and a clear homology between avidin and streptavidin (56), no sequence homology exists between the signal peptides of the two proteins.

Two ribosome binding sites preceded the initiator Met (positions 44-46) of the signal peptide. The distal site, CCACCT (positions 29-34), was representative of the consensus sequences observed for the ribosome binding site (44). However, it is atypically positioned fifteen nucleotides upstream from the initiator Met. The proximal binding site adjacent to the initiator Met, GCAGAG (positions 38-43), is a less typical sequence found in only approximately 10% of examined genes (44,45); its position is characteristic for this functional element. It is possible that the two adjacent ribosome binding sites enhance the interactions between ribosome and mRNA required for initiation of translation (45).

A typical polyadenylation signal (32) is located 65 nucleotides downstream from the carboxyl terminal amino acid (position 499), and 15 nucleotides prior to the poly (A) tail.

Expression of Avidin mRNA

Avidin mRNA was qualitatively examined by RNA blot hybridization (Northern) analysis. Diethylstilbestrol (DES) treatment causes proliferation and differentiation of the oviduct in immature chicks and synthesis of a number of egg-white proteins (46-50). Following hormone withdrawal, which causes cessation of oviduct growth and expression of egg-white proteins, the concentration of avidin mRNA was induced by a single injection of progesterone alone or with DES over a period of 8-24 hrs (Fig. 2). No avidin mRNA was detected in chicks treated acutely or chronically with DES. The size of mRNA corresponding to approximately 0.7 kb was detected in chicken oviduct total RNA using the 32 P-labeled 384 bp insert from λ cAV2. The exact length of the 5'-untranslated sequence in the mRNA is unknown at present.

Steroid hormone regulation and tissue specific distribution of the avidin mRNA was quantified by RNA dot hybridization analysis (Fig. 3). There was no effect of secondary stimulation by progesterone or estrogen on avidin mRNA level in the liver or kidney. Induction of the avidin mRNA by progesterone or by progesterone and estrogen was observed in the oviduct (Fig. 3, panel A). The concentration of oviduct GAPDH mRNA was monitored as an

Figure 4 Complexity of Avidin Sequences in Genomic DNA

Genomic DNA from the chick spleen (10 µg/lane) was digested to completion with Bam HI or Hind III, electrophoresed on a 1% agarose gel, transferred to a nitrocellulose filter and hybridized with the [³²P]-labeled cDNA insert from λcAV2. Restriction sites within the 374 bp λcAV2 insert are indicated at the top of the figure.

internal control, since the expression of the gene is constitutive and should not be affected by steroid hormone administration (40). As expected there was no effect of hormone treatment on this mRNA in any of the tissues examined (Fig. 3, panel B). Progesterone treatment resulted in an increase of avidin mRNA from an undetectable level to approximately 0.05 % (poly A RNA) at 24 hours.

Complexity of Avidin Sequences in Genomic DNA

Genomic DNA from chick spleen was examined by DNA blot hybridization

(Southern) analysis. DNA was digested to completion with Bam HI and Hind III enzymes and hybridized with the ^{32}P -labeled cDNA insert from λcAV2 . Only a single fragment was observed following Bam HI digestion, while two fragments were observed in the Hind III digest (Fig. 4). This is consistent with the interpretation that the avidin gene is encoded by a single copy gene.

Uses for a Complete cDNA Clone for Avidin

Isolation of a molecular probe coding for chicken avidin should be of use for studies of the selective regulation of a specific protein in reproductive tissue by progestins. Although certain other oviduct proteins are increased by progesterone, they are regulated also by estrogens and other steroid hormones (51-54). The hormone-independent phenomenon of avidin expression in diverse tissues during inflammation also can be studied more easily. There are few, if any, DNA probes to monitor the cellular response to inflammation at a molecular level. Finally, avidin has wide applications as a useful reagent for techniques as diverse as histochemistry and affinity column purification (55). Expression of this cDNA in an efficient vector system should provide a ready source of the natural molecule.

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