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CLONING AND EXPRESSION OF A PSEUDO-OVALBUMIN GENE

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SUMMARY

A pseudo-ovalbumin gene, bearing significant nucleotide sequence homology to the ovalbumin gene, has been cloned from genomic chick DNA. Similar to the authentic ovalbumin gene, the pseudo-gene is a unique sequence gene in the chick genome and is expressed at a low level in the immature chick oviduct. In contrast to the ovalbumin gene, expression of the pseudo-gene in the oviduct is not inducible by estrogen. The concentration of pseudo-gene RNA is only ~0.01% of that of authentic ovalbumin mRNA in estrogen-stimulated oviduct cells. Nucleotide sequence analysis of the two sequence related genes may reveal the molecular basis of differential response to steroid hormone induction in the same tissue.

INTRODUCTION

Using pOV230 (1), a recombinant plasmid containing an insert of full-length ovalbumin cDNA as a specific hybridization probe, the natural ovalbumin gene has been found to be contained within multiple Eco RI DNA fragments of the chick genome (2-5). Cloning and characterization of these ovalbumin gene-containing DNA fragments have led to the discovery that the structural ovalbumin gene is separated into 8 segments by 7 intervening sequences (6-15). In addition to the major Eco RI DNA bands containing fragments of the ovalbumin gene, a weak hybridization band at 3.2 kb of total Eco RI-digested chick DNA was also observed (6). This fragment was cloned using the λgtWES DNA vector (16) and appeared to be a pseudo-ovalbumin gene. The current report provides evidence for the existence of this pseudo-gene and reveals that it is expressed at a low level in chick oviduct cells, but is not under hormonal control.

MATERIALS AND METHODS

(a) Purification of the X3.2 DNA: Total chick DNA was digested to completion with Eco RI and fractionated on an RPC-5 column as described pre-

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viously (6). Individual fractions were analyzed by agarose gel electrophoresis followed by Southern hybridization (17) using [^{32}P]pOV230 as the hybridization probe (5). Fractions containing the 3.2 kb hybridization bands were pooled and the DNA was further enriched by preparative agarose gel electrophoresis (6).

(b) Cloning of the X3.2 DNA: The 100-fold enriched X3.2 DNA was ligated with the left and right arms of λgtWES DNA vector and the ligation mixture was cloned in E. coli LE392/ThyA in a P3 facility in accordance with the NIH guidelines. Thirty thousand recombinant phage plaques were screened for clones containing the X3.2 DNA by [^{32}P]pOV230 using a modified Benton and Davis procedure (18) as reported previously (6). Six positive clones were identified and cultured. The cloning of a similar X gene has been reported recently by Royal et al. (19).

(c) Electronmicroscopic analysis of the cloned X3.2 DNA: The X3.2 DNA was separated from the λgtWES arms by Eco RI digestion of the recombinants followed by preparative agarose gel electrophoresis. The DNA (10 μg/ml) was mixed with 100 μg/ml of total polyA-containing oviduct RNA in 70% formamide/ 10 mM EDTA/ 0.1 M NaCl/ 0.1 M Tris-HCl, pH 7.6. Total polyA-containing RNA was prepared from hen oviduct nucleic acid extract by repeated oligo dT-cellulose column chromatography. The mixture was sealed in capillary tubes and incubated at 80° for 5 min followed by 55° for 3 hr. The nucleic acid mixture was subsequently examined by an electron microscope as reported previously (9). For heteroduplex analysis, 10 μg/ml of the X3.2 DNA was incubated at 80° for 5 min followed by 25° for 3 hrs in the presence of 20 μg/ml of a previously cloned chick genomic segment containing the complete ovalbumin gene (9) in the same medium.

(d) Expression of X3.2 DNA as determined by R_0t analysis: Purified X3.2 DNA was labeled to about 2 x 10^7 cpm/μg using [^{3}H]dCTP and [^{3}H]TTP according to a modified method of Mackey et al. (20) as reported by Roop et al. (21). Isolation of RNA from chick oviduct nuclei and polysomes were as described previously (21). Hybridization of [^{3}H]X3.2 DNA with excess chick RNA or DNA was carried out at 68° in tapered vials in 0.6 M NaCl/2 mM EDTA/0.01 M HEPES, pH 7.0 for various lengths of time. Percent hybridization at every time point was determined by comparing trichloracetic acid resistant radioactivity before and after treatment with S1 nuclease (21).

RESULTS AND DISCUSSION

Cloning of the X3.2 DNA and its sequence homology with the chick ovalbumin gene are shown in Fig. 1. Using [^{32}P]pOV230 as the specific hybridization probe, the presence of a weakly hybridizable band at 3.2 kb can be observed readily in total Eco RI-digested chick DNA (Fig. 1, lane a). The other hybridization bands are the chick DNA segments containing fragments of the authentic ovalbumin gene (2-5). After amplification by molecular cloning, however, the X3.2 DNA showed significant sequence homology with the ovalbumin gene as indicated by the strong hybridization signal after digestion of the X3.2 clone DNA with Eco RI (Fig. 1, lane b).

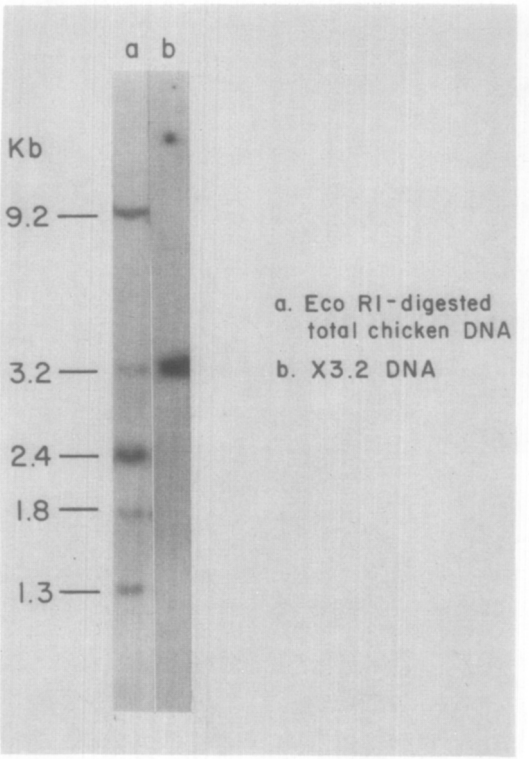

Figure 1 Radioautogram of Eco RI-digested total chick DNA (lane a) and the cloned X3.2 DNA (Lane b) after electrophoresis on a 1% agarose gel followed by Southern transfer and hybridization with [^{32}P] pOV230 DNA, which is a recombinant plasmid containing a full-length ovalbumin cDNA insert (1). Conditions of gel electeophoresis and Southern hybridization were as described previously (5).

Heteroduplex analysis was employed to determine the extent of sequence homology between the genomic X3.2 DNA and the chromosomal ovalbumin gene. We have recently reported the cloning of a chick DNA segment containing the entire ovalbumin gene (9). The two cloned genes were denatured thermally together and allowed to reassociate. Hybrid molecules were then examined by electron microscopy. A typical hybrid molecule and its corresponding line drawing are shown in Fig. 2A. It is apparent that the stable region of homology between the two genes is rather short. Numonic measurement has shown that this region is only 0.2-0.4 kb in length.

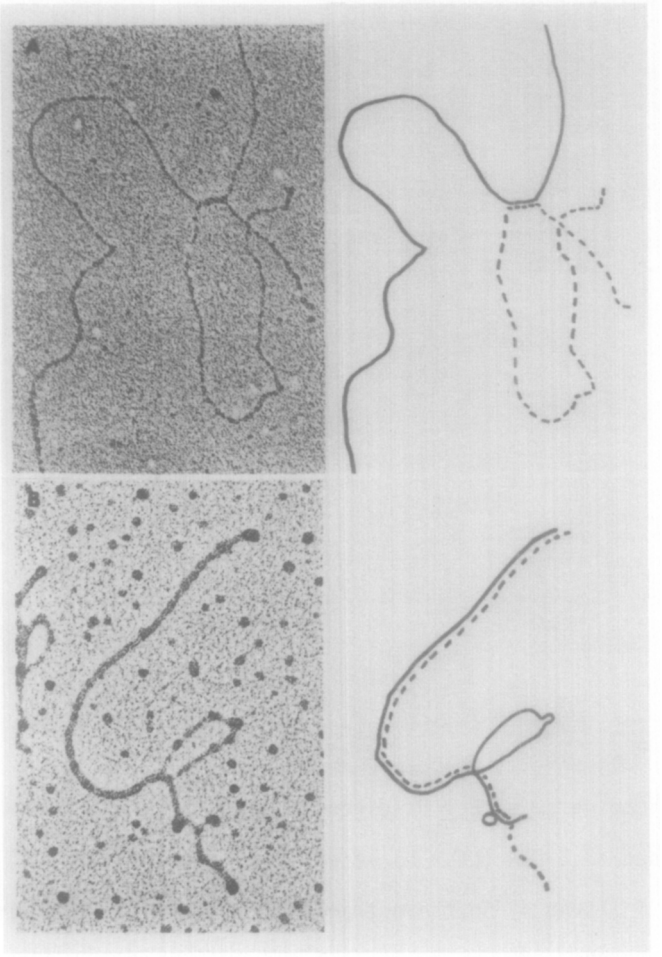

Figure 2 Electron micrographs and corresponding line drawings of the cloned X3.2 DNA: Panel A, heteroduplex analysis with Bam HI-linearized pOV12 DNA which is a recombinant plasmid containing the entire genomic chick ovalbumin gene (9); Panel B, after thermal denaturation and hybridization with total chick oviduct polyA-containing RNA. Conditions of hybridization and preparation of samples for electronmicroscopy were as described previously (9).

The messenger RNA coding region on the X3.2 DNA, if any, was next determined by electron microscopic analysis of hybrid molecules formed between the X3.2 DNA and total chick oviduct polyA-containing RNA (Fig. 2B). The hybridization conditions were such that only DNA/RNA hybridization could proceed but

not DNA/DNA reassociation. Thus, homologous regions between the single-stranded DNA and RNA would form duplexes, and intervening DNA sequences, if any, would be displaced as single-stranded loops between duplex regions. Such a hybrid molecule is shown in Fig. 2B. It is apparent that X3.2 DNA is not only expressed into a polyA-containing RNA species in the oviduct, but also contains a small and a large intervening sequence. Since the mRNA coding region on the X3.2 DNA is greater than 2 kb in length and its homologous region with the ovalbumin gene is only 0.2-0.4 kb in length, the X3.2 DNA cannot be a genotypic allele of the ovalbumin gene. In this regard, the X3.2 DNA appears to be similar to the X gene as reported recently by Royal et al. (19).

Since the X3.2 DNA is apparently a pseudo-ovalbumin gene and is expressed into mRNA in the oviduct, we have determined its gene frequency, hormone inducibility and tissue specificity by C_0t and R_0t analyses. The X3.2 DNA was labeled to high specific radioactivity and allowed to hybridize with excess chick DNA. Hybridization reached 70% with an equivalent $C_0t_{1/2}$ value of approximately 2×10^3 (Fig. 3A). The curve was superimposeable with that of the ovalbumin gene, indicating that the X3.2 DNA is also a unique sequence in the chick genome. The X3.2 DNA probe was then allowed to hybridize with RNA isolated from various chick sources (Fig. 3B). Insignificant hybridization with liver RNA has indicated that the pseudo-ovalbumin gene, similar to the ovalbumin gene, is not expressed in the liver to any appreciable extent. On the other hand, the pseudo-gene is apparently expressed at a low level in the oviduct of hormonally-withdrawn chicks as indicated by the high equivalent $R_0t_{1/2}$ value. The extent of expression of the X3.2 DNA is approximately 1 RNA molecule per oviduct cell nucleus, which is 30% of that of the ovalbumin gene in the same tissue in the absence of hormone. Although there is a thousand-fold induction in the expression of the ovalbumin gene by estrogenic stimulation (6,20), the level of expression of the X3.2 DNA has not increased to a significant extent as indicated by only a slight shift of the R_0t curve

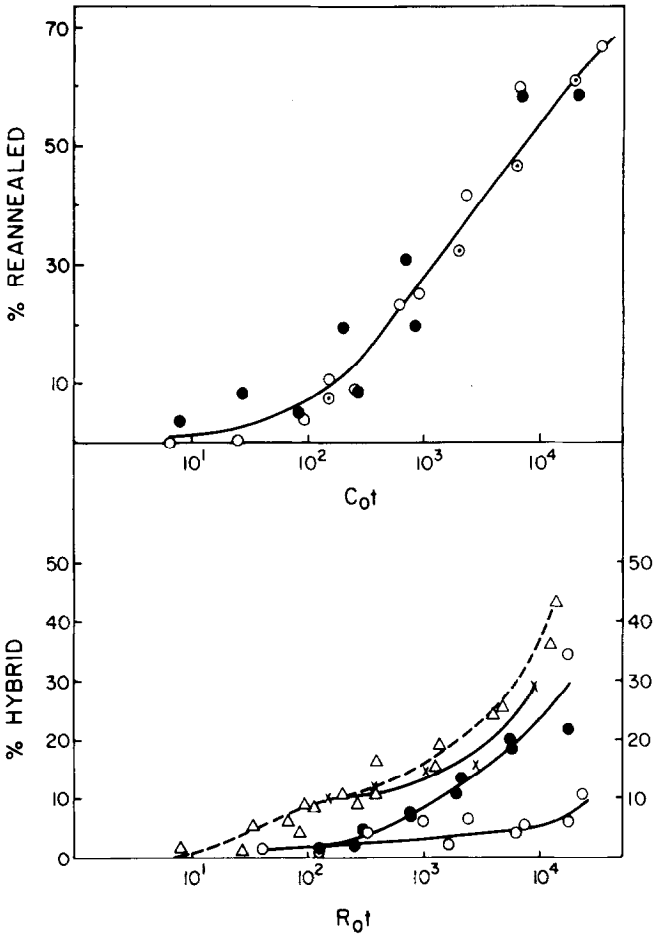

Figure 3 Hybridization of excess chick DNA and RNAs with the [^{3}H]labeled X3.2 DNA probe: Panel A, Hybridization between chick oviduct DNA with DNA probes prepared from X3.2 DNA (●), OV2.4 DNA (○) and $cDNA_{OV}$ (⊙). Conditions of hybridization and scoring of hybrid formations were carried out as described in Methods; Panel B, Hybridization between [^{3}H]labeled X3.2 DNA with nuclear RNAs isolated from hormone-stimulated chick oviduct (△), hormone-withdrawn chick oviduct (▲), chick liver (○), and polysomal RNA isolated from hormone-stimulated chick oviduct (X). Conditions of hybridization and scoring of hybrid formation were carried out as described in Methods.

to the left (Fig. 3B). The concentration of the pseudo-gene RNA is hence only ~0.03% of that of the authentic ovalbumin RNA in the hormonally stimulated oviduct nucleus (6,21). In addition, although the pseudogene transcript is present on oviduct polysomes and may be a messenger type RNA, its concentration is again only $\sim 10^{-4}$ of that of ovalbumin mRNA (Fig. 3B). Consequently, unlike the authentic ovalbumin gene, the expression of the ovalbumin pseudogene appears to be independent of the steroid hormone estrogen. It should be noted that this finding is in disagreement with that observed for the X gene by Royal *et al.* (19).

Due to the presence of sequence homology between the two genes, the fidelity of the hybrids observed in the R_0t curves must be verified by thermal denaturation analysis. The extents of hybridization of these R_0t curves have reached 40% which were much higher than could be expected from cross hybridization between the X3.2 DNA and authentic ovalbumin RNA. Since the homologous regions between the two genes are less than 15% of the length of X3.2 DNA, the extent of cross hybridization should not exceed 7.5% as only one DNA strand is usually expressed into RNA *in vivo* (21). Indeed there was a 5-10% hybridization between X3.2 DNA and estrogen-stimulated oviduct RNA at lower R_0t values; such a result was not obtained with the hormone-withdrawn chick RNA and could be the result of cross hybridization (Fig. 3B). This limited extent of cross hybridization was substantiated by the low melting temperature and broad spectrum of the initial 20% of the denaturation curve of the X3.2 DNA/RNA hybrids (Fig. 4). The remaining 80% of the hybrids melted at higher temperatures with a Tm of approximately 82° versus 85° for that of the ovalbumin cDNA/RNA hybrids. Thus, the great majority of the X3.2 DNA hybridization observed in the R_0t curves was not due to cross hybridization with authentic ovalbumin RNA.

Since the X and Y genes cloned by Royal *et al.* share some sequence homology with the ovalbumin gene, it has been suggested that duplications have occurred in the ovalbumin gene region in the course of evolution (19).

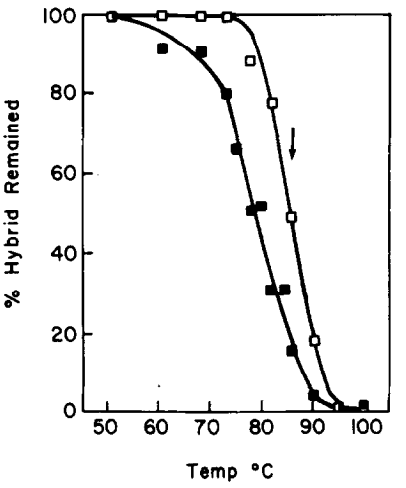

Figure 4 Thermal denaturation analysis of hybrids formed between hormonally stimulated chick oviduct nuclear RNA and [^{3}H]labeled X3.2DNA and $cDNA_{OV}$ probes. Hybridization of oviduct nuclear RNA to X3.2 DNA (■) and $cDNA_{OV}$ (□) were carried out to a R_0t of 5250 and 1750, respectively. Aliquots of the hybrids were then incubated for 5 min at indicated temperature followed by S1 nuclease treatment as described in Methods. Percent S1 resistant radioactivity was then plotted against the corresponding incubation temperature.

Although the existence of a pseudo-ovalbumin gene may shed some light on the derivation of the ovalbumin gene during evolution, the lack of response to hormone treatment is of great interest to us. What could be the molecular basis for a sequence related gene to be expressed in the same tissue and yet not regulated by a common mechanism? Nucleotide sequence analysis of the 5' termini of both genes would be a first step toward understanding this phenomenon and may enhance our appreciation of the molecular mechanism by which steroid hormones regulate eukaryotic gene expression.

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