Differential Hormonal Responsiveness of the Ovalbumin Gene and Its Pseudogenes in the Chick Oviduct†

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ABSTRACT: We describe the isolation of recombinant phages from a chicken gene library which contain two genes designated X and Y. These two genes are linked to the ovalbumin gene (OV) in the order 5′-X–Y–ovalbumin-3′ [Royal, A., Garapin, A., Cami, B., Perrin, F., Mandel, J. L., LeMeur, M., Bregegegre, F., Gannon, F., LePennec, J. P., Chambon, P., & Kourilsky, P. (1979) *Nature (London) 279*, 125–132]. Both genes contain multiple intervening sequences and share limited sequence homology with the authentic ovalbumin gene but are expressed in oviduct cells at different levels. X and Y hybridization probes were prepared in order to study the expression and the relative hormonal responsiveness of these three genes in the chicken oviduct. The sequence specificity of the probes was demonstrated by Southern hybridization assays. Northern hybridization studies using the X and Y gene probes indicated the presence of putative precursor molecules in stimulated oviduct ribonucleic acid preparations, which differ in size from those observed for ovalbumin. R_0t analysis has demonstrated that, similar to the ovalbumin gene, the level of X and Y gene transcripts is increased by the steroid hormone estrogen, but to varying degrees. The extent of hormonal responsiveness of the three closely related genes is in the order (normalized) relative to ovalbumin of OV:Y:X $\simeq$ 100:10:1. Pulse-labeling studies of these three closely linked genes suggest that in estrogen-stimulated oviduct, the markedly different steady-state levels of the X, Y, and ovalbumin gene transcripts reflect their differential transcription rates.

The chicken ovalbumin gene, the expression of which is regulated by steroid hormones, is comprised of eight structural gene segments separated by seven intervening sequences (Woo et al., 1978; Dugaiczyk et al., 1979; Breathnach et al., 1978; Gannon et al., 1979). We have previously reported the isolation from a chicken gene library (Dodgson et al., 1979) of a recombinant phage λ4A·OV which contains the entire ovalbumin gene (Dugaiczyk et al., 1979). Using the S1 nuclease mapping method of Berk & Sharp (1977), we have determined that the 5′ and 3′ ends of ovalbumin precursor RNA[1] are coincident with the capping and polyadenylation sites on the gene (Roop et al., 1980). Northern hybridization and pulse–chase experiments have also supported a model of mRNA maturation in which the entire ovalbumin gene is transcribed as a large precursor RNA molecule which is subsequently processed by excision of intervening sequence transcripts and proper ligation of structural sequence transcripts (Roop et al., 1978; Tsai et al., 1980).

Recently, Royal et al. (1979) and subsequently Woo et al. (1979) have demonstrated that related genes of unknown function but bearing sequence homology with the ovalbumin gene exist in the chicken genome. Two genes, designated X and Y, are transcriptionally active in the chick oviduct. The ovalbumin gene and the X and Y "pseudogenes" are closely linked at the same chromosomal locus with the linkage order 5′-X–Y–ovalbumin-3′, and all three genes appear to be induced by estrogen (Royal et al., 1979). It was thus of immediate interest to determine whether the expression of the X, Y, and ovalbumin genes is coordinantly regulated by steroid hormones. The preparation of gene-specific hybridization probes and their utilization in quantitating the degree of hormonal responsiveness of the X, Y, and ovalbumin genes is the subject of this paper.

Materials and Methods

Materials

Oviducts were obtained from White Leghorn chicks. In the case of stimulated oviducts, chicks were implanted weekly with a 20-mg diethylstilbestrol (DES) pellet subcutaneously (Sigma Chemical Co.), which provided continuous release of DES for 8–9 days. In the case of withdrawn oviducts, chicks were subcutaneously injected daily with 2.5 mg of diethylstilbestrol for 14 days and then withdrawn from all hormone for 14 days. For experiments involving acute stimulation with estrogen, chicks received an injection of 2.5 mg of DES on the 14th day of withdrawal and then 24 h later. Oviducts were collected at the indicated time intervals. Restriction endonucleases were purchased from Bethesda Research Laboratories. S1 nuclease was obtained from Miles Laboratories. DNA polymerase I was a product of Boehringer. [α-^{32}P]dCTP, [α-^{32}P]dTTP, and [^{3}H]dCTP were purchased from Amersham. All chemicals were reagent grade and were purchased from Fisher Scientific Co. A chicken gene library constructed by inserting random chicken DNA fragments into Charon λ4A DNA was kindly provided by Drs. Engel, Dodgson, Axel, and Maniatis (Dodgson et al., 1979).

Methods

Screening of the Chicken Gene Library. All cloning and propagation procedures were carried out in a certified P3 facility in accordance with National Institutes of Health guidelines for recombinant DNA research. The phage titer of the chicken gene library was determined by serial dilution, and ~10000 phages were spread in soft agar onto each of 200 square agar plates. Screening of the recombinant phage plaques was carried out by a modification of the procedure of Benton & Davis (1977) as described by Woo (1979). The

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; cRNA, complementary RNA; DNA, deoxyribonucleic acid; cDNA, complementary DNA; BSA, bovine serum albumin; SSC, standard saline citrate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; poly(A), poly(adenylic acid); DBM, diazobenzyloxymethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/80/0419-5586$01.00/0

hybridization probes used for screening were DNA fragments prepared from the extreme 5′ termini of the chicken DNA inserts in recombinant phages CL26 and CL78 (described in the text). The probes are unique chick DNA sequences and were prepared by complete digestion of CL26 DNA with *Bam*HI and CL78 DNA by *Eco*RI, followed by extraction of a 2.1-kilobase (kb) fragment and a 2.5-kb fragment, respectively, from preparative 1.0% agarose slab gels as previously described (Lai et al., 1979a). The labeling of this DNA fragment with ^{32}P was carried out by the nick translation procedure of Maniatis et al. (1975) as modified in our laboratory (Lai et al., 1979a).

DNA Transfer and Southern Hybridization Analysis. Following restriction endonuclease digestion, DNA fragments were separated by agarose slab gel electrophoresis and transferred to nitrocellulose filters by the procedure of Southern (1975). After transfer, the filters were dry baked for 2 h at 68 °C, followed by incubation (68 °C, 6 h) in a 6 × SSC solution containing 0.04% ficoll, 0.04% poly(vinylpyrrolidone), and 0.04% BSA (Denhardt, 1966). Hybridization with a ^{32}P-labeled probe was carried out in the same solution containing 0.5% $NaDodSO_4$ and 1 mM EDTA for 12 h at 68 °C. Washing of the filters was carried out at 68 °C with three 2-h washes in 1 × SSC and 0.5% $NaDodSO_4$, and a 1-h wash in 1 × SSC without $NaDodSO_4$. The filters were then exposed to X-ray film by using Du Pont cronex intensifying screens at −20 °C.

Electrophoresis of RNA and Transfer to DBM Paper for Hybridization. Poly(A)-containing RNA was prepared from stimulated and withdrawn chick oviduct by extraction of total nucleic acids from tissues with phenol, followed by oligo(dT)–cellulose column chromatography as described previously (Woo et al., 1975). The procedure for agarose gel electrophoresis in the presence of methylmercury hydroxide (Bailey & Davidson, 1976) and the transfer of RNA to DBM and hybridization to [^{32}P]DNA probes were carried out by a modified procedure of Alwine et al. (1977) as described by Roop et al. (1978).

RNA Excess Hybridization Analysis. The DNA probes were labeled with ^{3}H as described by Roop et al. (1978). RNA–DNA hybridization reactions were performed in Kontes reaction vials containing a 600–700-cpm [^{3}H]DNA probe (10×10^6–40×10^6 cpm/μg) and 1–150 μg of RNA in a 50-μL solution for various lengths of time. Following incubation, the samples were treated with S1 nuclease, and the trichloroacetic acid precipitable, S1-nuclease resistant, radioactive counts were determined and plotted as previously described (Tsai et al., 1978; Roop et al., 1978).

Determination of Transcription Rates in Oviduct Tissue Suspensions. Preparation of oviduct tissue suspensions and extraction of RNA were carried out as previously described (Tsai et al., 1980; Swaneck et al., 1979). Incorporation of [^{3}H]uridine and [^{3}H]cytidine was carried out at 41 °C in F-12 medium supplemented with 10^{-8} M estradiol, 1 μg/mL insulin, and 10^{-8} M dexamethasone. Preparation of plasmid DNA-bearing filters was carried out as described by Tsai et al. (1978). Hybridization reactions were carried out in 0.6 M NaCl, 0.01 M Hepes (pH 7.0), and 2 mM EDTA at 68 °C for 18 h. Washing of the filters prior to determination of hybridized radioactive counts was carried out as described by Tsai et al. (1980), and preparation of cRNA from recombinant plasmid templates was carried out as described by Roop et al. (1978).

Electron Microscopic Analysis of DNA–RNA Hybrid Molecules. DNA–RNA hybrids were formed by incubation of 10 μg/mL DNA with 1–200 μg/mL RNA in 70% formamide, 10 mM EDTA, 0.1 M NaCl, and 0.1 M Tris-HCl, pH 7.6. The mixture was sealed in capillary tubes and incubated at 80 °C for 5 min, followed by incubation at 55 °C for 3 h. The incubated samples were immediately prepared for electron microscopic analysis as described previously (Dugaiczyk et al., 1979) and viewed with a Joel 100C electron microscope.

Results

Isolation of X and Y Pseudogenes from a Chicken Gene Library. The chicken DNA fragment within the recombinant phage λ4A·OV contains the entire ovalbumin gene plus approximately 5.2 and 6.2 kilobases (kb) of DNA flanking the 5′ and 3′ termini of the gene, respectively (Dugaiczyk et al., 1979). In addition to λ4A·OV (originally designated CL64), other ovalbumin gene-containing recombinant phages were also obtained and characterized by restriction endonuclease mapping. One of these recombinants, designated CL26, contained additional sequences flanking the 5′ end of the ovalbumin gene. Some of the restriction sites of these cloned DNAs are presented in Figure 1. As depicted in the figure, the chicken DNA insert in CL26 extended the range of DNA ~11 kb beyond the 5′ terminus of the ovalbumin gene. A *Bam*HI-generated 2.1-kb fragment from CL26 DNA was ~ 9 kb from the 5′ terminus of the ovalbumin gene and was utilized as a probe to isolate clones from the same chick gene library containing chick DNA sequences further 5′ from the ovalbumin gene. Several positive phages were identified and their DNAs characterized by restriction endonuclease mapping. The map of one of these clones, designated CL78, is presented in Figure 1. The inserted DNA in this clone extended the length of DNA ~25 kb beyond the 5′ terminus of the ovalbumin gene, a sufficient length to contain all of the Y gene and a portion of the X gene sequences as reported by Royal et al. (1979).

In order to obtain the entire X gene sequence, we employed the 5′ terminal 2.5-kb *Eco*RI fragment of CL78 DNA as a probe for a second round of library screening. One of the recombinant phages, CL36, contained 16 kb of chick DNA sequences further 5′ from the ovalbumin gene and should contain the entire X gene (Figure 1). These clones, all containing unique sequence DNA, represent a single contiguous portion of the chromosome extending 39.6 kb 5′ of the ovalbumin gene.

Molecular Structure of Y and X Pseudogenes. Demonstration of the presence of the Y and X mRNA coding regions in CL78 and CL36 DNAs was accomplished by electron microscopic mapping after hybridization with total poly(A)-containing RNA extracted from hormonally stimulated chick oviducts. Figure 2 shows representative molecules of the Y and X gene structures. For comparison purposes, a molecule of the chromosomal ovalbumin gene is also shown. A total of seven single-stranded loop regions are evident in all three molecules as indicated on the corresponding line drawings. Such structures are routinely observed for the Y and X gene hybrids, indicating that the structural sequences of the Y and X genes, like the ovalbumin gene, are organized into eight segments separated by seven intervening sequences. The Y gene is ~6.3 kb in length and is separated from the ovalbumin gene by 8.7 kb of spacer DNA. The sizes and locations of intervening sequences are similar to those reported by Royal et al. (1979). The X gene is located 5.4 kb further 5′ from the Y gene. The 3′-terminal structure of the X gene containing intervening sequences F and G is again in general agreement with that reported by Royal et al. (1979). The entire X gene is 8.2 kb in length, and there are 10 kb of additional DNA flanking its 5′ terminus in CL36 DNA. The striking similarity

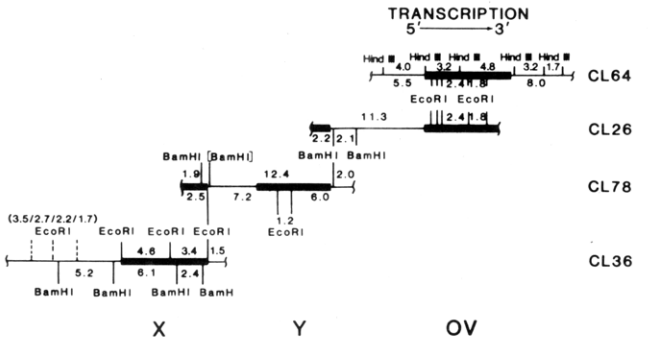

FIGURE 1: Restriction maps of four recombinant phages isolated from a chicken gene library containing overlapping DNA regions of the ovalbumin gene locus in the chicken genome. The linear DNA maps of individual phages were constructed on the basis of restriction endonuclease digests with *Bam*HI, *Eco*RI, and both enzymes together. The positions of these sites and the distance in kilobases are presented in the figure. The synthetic *Eco*RI sites used in construction of the recombinant phages are indicated by (∫), and the positions of the authentic genes and pseudogenes in individual phages are shown by (■). Restriction mapping of CL64 DNA had been reported previously (Dugaiczyk et al., 1979). The restriction maps of CL26, CL78, and CL35 DNAs were established as the following (data not shown): *Eco*RI digestion of CL26 DNA yielded chicken DNA inserts of 11.3, 2.4, 1.8, 0.5, and 0.1 kb in length. The 2.4-, 1.8-, and 0.5-kb fragments were internal fragments of the ovalbumin gene. The 11.3- and 0.1-kb fragments were created by synthetic *Eco*RI linkers during construction of the phage library (Dodgson et al., 1979). The 11.3-kb fragment corresponded to the 5′ end of the ovalbumin gene since it hybridized to a probe containing only the 5′ half of the ovalbumin gene. This fragment was cleaved by *Bam*HI twice to yield three fragments of 7, 2.2, and 2.1 kb in length. Since a *Bam*HI site had previously been mapped at 7 kb 5′ from the ovalbumin gene (Lai et al., 1978) and only the 2.1-kb fragment could be released internally from CL78 DNA by *Bam*HI digestion, the two *Bam*HI sites in the chicken DNA insert of CL26 must be as shown in the figure. CL78 DNA was cleaved by *Eco*RI into four inserted fragments of 7.2, 6.0, 2.5, and 1.2 kb in length (Figure 4A, lane 4). The 6.0-kb fragment was digested with *Bam*HI into 4.0- and 2.0-kb fragments, and only the latter hybridized with the 2.1-kb *Bam*HI fragment from CL26 DNA, providing the overlapping regions between the two clones. The 4.0-kb fragment hybridized with an ovalbumin DNA probe and must contain part of the Y gene. *Bam*HI digestion of CL78 DNA released an internal 12.4-kb chicken DNA fragment which could be cleaved by *Eco*RI into three fragments of 6.8, 4.0, and 1.2 kb in length. Since the 7.2-kb *Eco*RI fragment was the only one large enough to yield a 6.8-kb fragment by *Bam*HI digestion, the 1.2-kb *Eco*RI fragment must be internal within the 12.4-kb *Bam*HI fragment and is located between the 7.2- and 6.0-kb *Eco*RI fragments. The 2.5-kb *Eco*RI fragment, therefore, must be located at the 5′ end of the clone, and indeed this fragment was cut by *Bam*HI into 1.9- and 0.6-kb fragments that were both hybridizable to a 3.2-kb *Eco*RI fragment containing part of the X gene (Woo et al., 1979). These results dictated that there must be an internal 1.0-kb *Bam*HI fragment in CL78 DNA, which was indeed observed (Figure 4A, lane 5). It should be noted that the [*Bam*HI] site in CL78 DNA is not present in some of the other clones and is polymorphic. Such genotypic polymorphism had previously been observed in the chromosomal ovalbumin gene (Weinstock et al., 1978; Lai et al., 1979b) and in the globin gene system (Jeffreys, 1979; Kan & Dozy, 1978). CL36 DNA contained 19.6 kb of chicken DNA insert which was released from the vector as seven *Eco*RI fragments of 4.6, 3.5, 3.4, 2.7, 2.2, 1.7, and 1.5 kb in length (Figure 4A, lane 7). The 3.4-kb fragment hybridized with the 2.5-kb *Eco*RI fragment of CL78 DNA, providing the overlapping regions between the two clones. This fragment contained an internal 2.4-kb *Bam*HI fragment which, together with the neighboring 6.1-kb *Bam*HI fragment, hybridized with the previously cloned 3.2-kb X gene fragment (Woo et al., 1979). Since the 4.6-kb *Eco*RI fragment could be released from the 6.1-kb *Bam*HI fragment, this *Eco*RI fragment must also be neighboring the 3.4-kb *Eco*RI fragment. Indeed, both of these fragments contained X gene sequences since they both hybridized to an ovalbumin DNA probe. The 1.5-kb *Eco*RI fragment hybridized with the 7.2-kb *Eco*RI fragment of CL78 DNA and must have been created by an artificial *Eco*RI site in the phage. The remaining *Eco*RI fragments are located 5′ from the X gene. Since the exact positions have not yet been mapped, the *Eco*RI sites harboring these fragments in CL36 DNA are indicated by dashed lines.

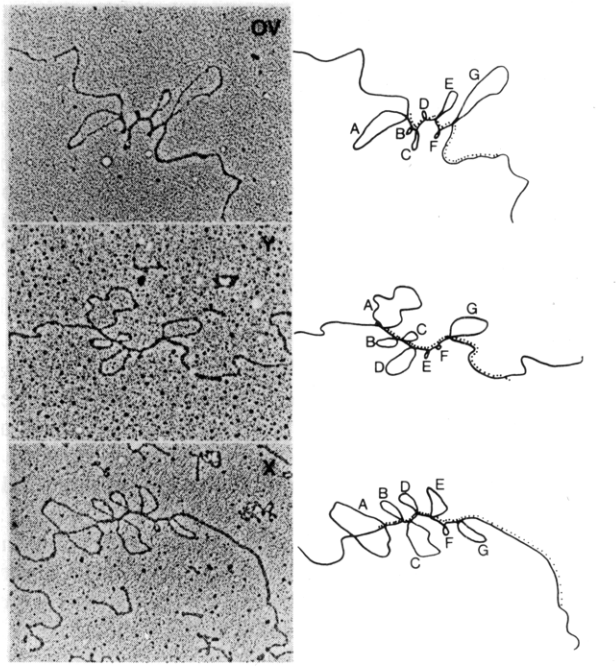

FIGURE 2: Electron micrographs and corresponding line drawings of hybrid molecules formed between poly(A)-containing RNA from stimulated chick oviduct and denatured CL64 DNA (upper panel), CL78 DNA (middle panel), and CL36 DNA (lower panel). (—) Single-stranded DNA; (···) oviduct RNA. Hybridization was carried out under conditions that favored the formation of RNA–DNA hybrids but not renatured DNA molecules. Homologous regions between DNA and RNA molecules would form hybrids, and intervening regions within DNA molecules not represented in the RNA molecules would be displaced to form single-stranded DNA loops. A total of seven loops designated A, B, C, D, E, F, and G are apparent within the ovalbumin and X genes as shown in the upper and lower panels. In the Y gene there are two small intervening loops (E and F) in addition to the five apparent loops (A, B, C, D, and G) as shown in the middle panel. The two small loops are present at the same locations in 10 individual molecules.

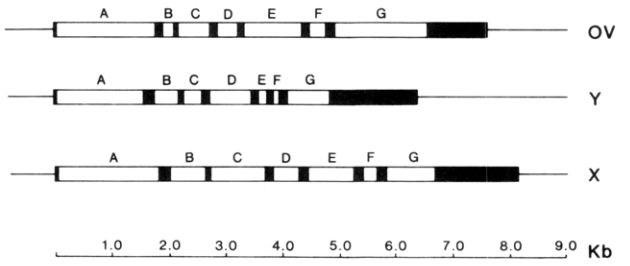

FIGURE 3: Molecular structure of authentic and pseudo ovalbumin genes as determined by electronmicroscopy. (■) Structural gene regions; (□) intervening DNA regions. The scale is shown in kilobase pairs (kb), and the absolute size of each gene was calculated from internal standards of ϕX174 DNA in the same electron micrographs. The sizes of individual duplexed and looped regions of each gene were determined from 10 independent molecules by using a neumonic map measure. The average of the independent determinants was then used to construct the molecular structure of each gene. Individual measurements varied no more than 10% of the mean.

in sequence organization between these three linked genes as determined by electron microscopic mapping is shown in Figure 3.

A *Bam*HI-generated 12.4-kb fragment from CL78 DNA (Figure 1) that contains the entire Y gene region was subcloned in the *Bam*HI site of the plasmid vector pBR322 (pY12.4) to facilitate propagation of the Y gene sequences. Similarly, a 8.0-kb DNA fragment containing the majority of X gene sequences was subcloned in the *Eco*RI site of pBR322 (pX8.0). A 12-kb DNA fragment containing the

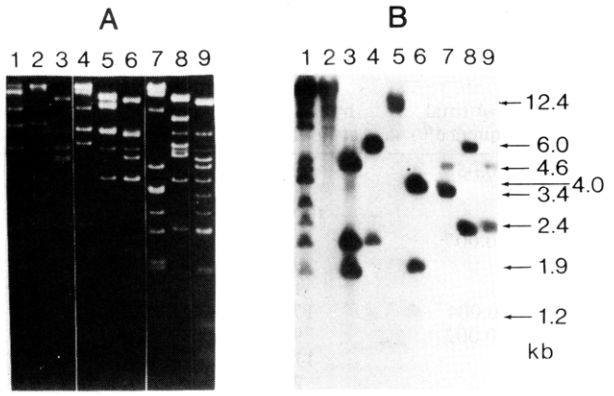

FIGURE 4: Sequence homology between authentic and pseudo ovalbumin genes by Southern hybridization. (Panel A) Ethidium bromide stain of an agarose gel containing CL64 (lanes 1–3), CL78 (lanes 4–6), and CL36 DNA (lanes 7–9) after digestion with *Eco*RI (lanes 1, 4, and 7), *Bam*HI (lanes 2, 5, and 8), or both enzymes (lanes 3, 6, and 9). (Panel B) Radioautogram of a Southern blot of the gel shown in panel A using as the hybridization probe ^{32}P-labeled pOV230 DNA, which is a full-length ovalbumin cDNA clone (McReynolds et al., 1977).

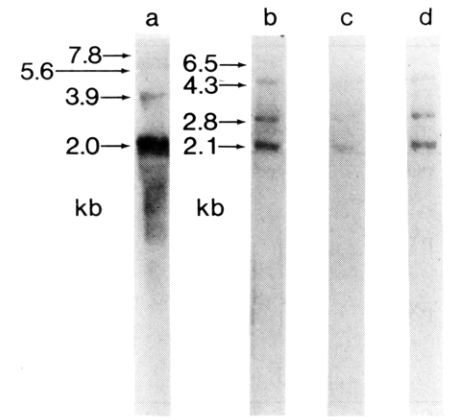

FIGURE 5: Radioautographic detection of authentic and pseudo ovalbumin gene precursor and mRNAs in stimulated oviduct RNA preparations. 20 μg of nuclear RNA from estrogen-stimulated chick oviducts was separated by size on methylmercury hydroxide gels and transferred to DBM paper for hybridization. The probes were labeled to about 1×10^8–2×10^8 cpm/μg, and 1×10^7 cpm (100 ng) was utilized for each hybridization. Hybridization was carried out with either the gene-specific probes alone, in the presence of 300 μg of denatured homologous plasmid DNA, or 300 μg each of the two denatured heterologous plasmids. (Lane a) pOV12 probe alone; (lane b) $Y_{1.2}$ probe alone; (lane c) $Y_{1.2}$ probe plus unlabeled pY12.4 DNA; (lane d) $Y_{1.2}$ probe plus unlabeled pOV12 and pX8.0 DNAs.

entire ovalbumin gene had previously been obtained from CL64 DNA by partial *Hin*dIII digestion and cloned in the *Hin*dIII site of pBR322 (pOV12).

Regions of Homology between X, Y, and Ovalbumin Gene Sequences. Regions of sequence homology existing between the X, Y, and ovalbumin gene sequences were detected by Southern hybridization. CL64, CL78, and CL36 DNAs were digested to completion with either *Eco*RI or *Bam*HI or both enzymes. The resulting DNA fragments were separated by agarose gel electrophoresis followed by transfer to nitrocellulose paper. Hybridization was carried out with ^{32}P-labeled ovalbumin DNA fragments of pOV230, which is a recombinant plasmid containing a full-length ovalbumin cDNA insert (McReynolds et al., 1977). Strong hybridization signals were obtained not only with the authentic ovalbumin gene fragments (Figure 4, panel B, lanes 1–3) but also signals of similar strength were obtained with various DNA fragments containing the Y and X genes (Figure 4, panel B, lanes 4–9). There is, thus, significant sequence homology in the structural segments between the authentic ovalbumin gene and the X and Y pseudogenes in the same chromosomal locus within the chick genome. When the experiment was repeated by using as the hybridization probe labeled pOV12 DNA, which contains all of the intervening sequences in addition to the structural ovalbumin gene sequences, the hybridization signals obtained from the Y and X DNA fragments were relatively much weaker (not shown). Thus, homologous sequences between the three genes appear to be mainly located within the structural gene segments. Furthermore, the homology is also confined within certain regions on the X and Y genes. Specifically, the 1.2-kb *Eco*RI fragment of the Y gene and the 4.6-kb *Eco*RI fragment of the X gene hybridized only weakly with the ovalbumin DNA fragments (Figure 4, lanes 4 and 7). These fragments were thus utilized as specific probes to detect Y and X gene transcripts in the oviduct cell.

Expression of X, Y, and Ovalbumin Genes in the Chick Oviduct. Comparative studies of expression of the X, Y, and ovalbumin gene transcripts were initiated by Northern hybridization. Total poly(A)-containing RNA extracted from estrogen-stimulated chick oviducts was electrophoresed in triplicate on an agarose slab gel containing methylmercury hydroxide. Following electrophoresis, the RNA was transferred to diazobenzyloxymethyl paper and hybridized separately with ^{32}P-labeled 4.6-kb X DNA ($X_{4.6}$), 1.2-kb Y DNA ($Y_{1.2}$), or ovalbumin gene probes.

Because of the abundance of ovalbumin gene transcripts within RNA preparations from stimulated oviducts, slight contamination of the X and Y gene specific probes by other restriction fragments bearing sequence homology with the ovalbumin gene would have resulted in preferential detection of ovalbumin gene transcripts in the Northern hybridization assay. To minimize possible errors caused by this problem, we tested three identical RNA strips with each hybridization probe in the absence or presence of a large excess of unlabeled DNA competitors. Each RNA-filter strip received 10^7 cpm of a given ^{32}P probe, and hybridization to RNA-filter strip 1 was carried out in the absence of competitor DNA. For RNA strip 2, hybridization was carried out in the presence of a large excess of unlabeled homologous competitor DNA. For RNA strip 3, hybridization was carried out in the presence of a large excess of each of the unlabeled heterologous competitor DNAs. The resulting radioautographic data obtained are presented in Figure 5.

The results obtained with the [^{32}P]ovalbumin probe are shown in lane a. Consistent with previous reports (Roop et al., 1978; Tsai et al., 1980), characteristic precursor ovalbumin RNA bands of 7.8 kb, 4.75 kb, and 3.45 kb and a mature mRNA band of 2.05 kb are present in the stimulated oviduct RNA. Results obtained with the [^{32}P]$Y_{1.2}$ probe are shown in lane b. As was the case with the ovalbumin gene probe, mRNA and precursor hybridization signals were detected in stimulated oviduct RNA preparations. That the detected bands represent Y gene specific signals and not cross-reaction with ovalbumin or X gene transcripts is indicated by the fact that the hybridization signals display significant competition by pY12.4 DNA (lane c) but not with pOV12 and pX8.0 DNAs (lane d). The sizes of the Y-specific precursor and mRNA bands also differ slightly from those of ovalbumin, being 6.7, 4.3, 2.8, and 2.1 kb, respectively. The size of the largest putative Y precursor RNA agrees with the size of the Y gene measured by electron microscopy. Under parallel hybridization conditions, specific hybridization signals at 3.6 and 2.4 kb were obtained with the [^{32}P]$X_{4.6}$ probe, but only after prolonged radioautography (not shown). The concen-

Table 1: Hybridization of [^{3}H]RNA Synthesized in Oviduct Tissue Slices to DNA Filters

filter[a]	cRNA competitor[b]	cpm hybridized after pulse[c]	% of ^{32}P internal standards remaining after competition	% of total sequence[d]	cpm hybridized after chase[e]	% of ^{32}P internal standards remaining after competition
pOV12		4418	100.0	0.058	926	100.0
	pOV12	480	9.9		110	6.4
	pY12.4	4550	82.5		1026	75.8
pY12.4		524	100.0	0.007	114	100.0
	pOV12	302	83.1		90	76.1
	pY12.4	118	10.2		10	12.7
pX8.0		292	100.0	0.004	176	100.0
	pOV12	174	63.0	0.002	94	51.2
	pY12.4	244	87.5		112	84.5
	pX8.0	54	8.4		20	7.3

[a] 7.5×10^6 cpm was used in every hybridization. [b] 15 μg of competitor RNAs transcribed from various plasmid DNAs were used. [c] [^{32}P]-RNAs transcribed from various plasmid DNAs were used as internal standards to monitor the recovery of hybridizable sequence. The recovery in this experiment was ~50%. [d] Each experiment has been carried out at least twice. The range of variation for a series of independent samples is ±20%. [e] Chase was for 30 min after addition of 5 mM uridine and cytidine.

tration of larger precursor RNA was too low to be detected in this experiment.

Differential Hormonal Responsiveness of X, Y, and Ovalbumin Genes. Having demonstrated the expression of the X, Y, and ovalbumin genes in the oviduct, it was important to determine the hormonal responsiveness of these linked and closely related genes. The relative concentrations of transcripts from each of the genes in total nuclear RNA preparations were quantitated by using estrogen-stimulated and withdrawn chick oviducts. Presented in Figure 6 are results obtained from hybridization studies by R_0t analysis using ^{3}H-labeled ovalbumin, $Y_{1.2}$, and $X_{4.6}$ probes. Relative transcript abundance in the RNA preparation was calculated on the basis of observed $R_0t_{1/2}$ values. Consistent with previous hybridization studies (Roop et al., 1978), a dramatic shift in $R_0t_{1/2}$ values was obtained with the [^{3}H]ovalbumin probe between stimulated ($R_0t_{1/2} = 1.2 \times 10$) and withdrawn ($R_0t_{1/2} \simeq 5 \times 10^3$) RNA preparations (upper panel). These values are indicative of an estrogen-mediated increase in the concentration of nuclear ovalbumin gene transcripts in withdrawn vs. stimulated chick oviducts from <1 to 240 molecules per cell nucleus, respectively.

A similar, though less dramatic, shift in the concentration of Y and X gene transcripts within the same stimulated and withdrawn oviduct nuclear RNA preparations was observed. The $R_0t_{1/2}$ values obtained with the [^{3}H]$Y_{1.2}$ probe in stimulated ($R_0t_{1/2} = 2.8 \times 10^2$) and withdrawn ($R_0t_{1/2} = 6 \times 10^3$) RNA preparations are consistent with Y gene transcript concentrations of 12 and <1 molecule(s) per oviduct cell nucleus, respectively (middle panel). Similarly, the $R_0t_{1/2}$ values using the $X_{4.6}$ DNA probe were 0.55 and 10^4 for stimulated and withdrawn RNA (lower panel), corresponding to concentrations of 2–3 and <1 molecule(s) per cell, respectively.

It should also be noted that the concentrations of Y and X gene transcripts measured with the gene-specific hybridization probes do not differ significantly from values obtained by using the entire Y and X gene as the hybridization probe, except for a generally higher level of background hybridization due to limited sequence homologies among the X, Y, and ovalbumin genes (data not shown). This indicates that the differential transcript concentrations observed in these studies represent true steady-state levels of the three gene sequences and not differential accumulation levels of probe-specific transcripts.

Transcription Rates of X, Y, and Ovalbumin Genes. As demonstrated above, the steady-state nuclear concentrations of X, Y, and ovalbumin gene transcripts are quite different.

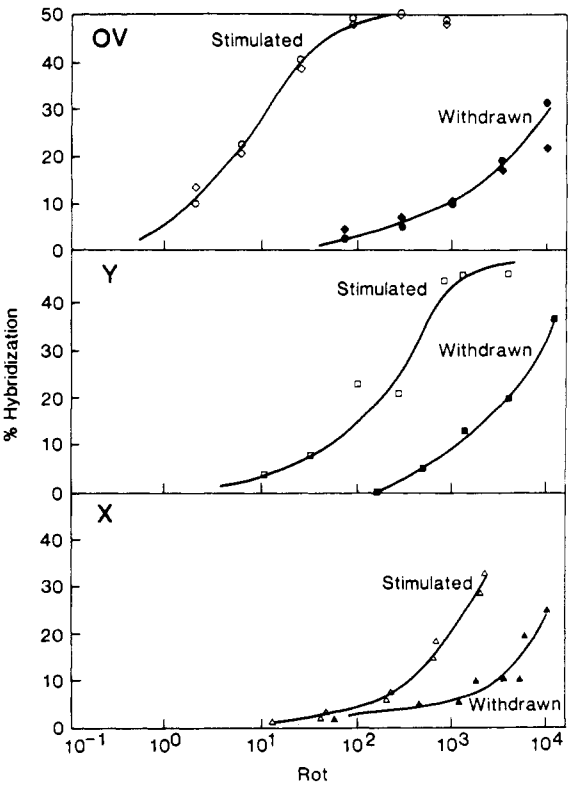

FIGURE 6: Steady-state levels of X, Y, and ovalbumin gene transcripts in estrogen-stimulated (open symbols) and withdrawn (closed symbols) oviduct nuclear RNA preparations. ^{3}H probes used in the upper panel were the *Eco*RI-generated 1.8-kb (◇) and 2.4-kb (○) fragments of the ovalbumin gene (Dugaiczyk et al., 1979), the 1.2-kb *Eco*RI fragment of the Y gene (□) in the middle panel, and the 4.6-kb *Eco*RI fragment of X gene (△) in the lower panel.

Such observed differences could be due to a differential rate of turnover and/or a differential rate of gene transcription. In order to determine the relative transcription rate of these three genes, we suspended hormonally stimulated oviduct tissue in a defined medium and allowed it to incorporate [^{3}H]uridine and [^{3}H]cytidine for a brief period (7.5 min). The ^{3}H-labeled nuclear RNA was then extracted and hybridized to filters containing either pOV12, pY12.4, or pX8.0 DNA by procedures previously described (Tsai et al., 1980; Roop et al., 1978). As a means of assessing the percent recovery of potentially hybridizable material bound to the filter, [^{32}P]cRNA transcribed in vitro from pOV12, pY12.4, and pX8.0 DNA was added to the hybridization reactions as an internal

standard. The routine recovery of ^{32}P internal standard also allowed a means of assessing the specificity of the hybridization reaction. As indicated in Table I, [^{32}P]cRNA transcribed from each of the recombinant plasmid templates hybridized well with DNA filters containing homologous DNA. When excess unlabeled cRNA from the ovalbumin and Y gene bearing plasmids was added as a competitor in the hybridization reaction, only the homologous competitor cRNA was capable of significantly reducing the hybridized counts. There was, however, substantial hybridization between X gene transcript and ovalbumin DNA due to sequence homology, as indicated by the competition value of 37% for [^{32}P]cRNA synthesized from X8.0 DNA.

The hybridization values obtained with the ^{3}H pulse-labeled nuclear RNA preparations are summarized in Table I. It is clear that significantly more label has been incorporated into ovalbumin transcripts than into Y and X gene transcripts. The apparent level of incorporation into X gene transcripts must be corrected for cross-hybridization with the ovalbumin gene, and the final level after competition with ovalbumin cRNA should be a more accurate estimate. Given the gene-specific ^{3}H counts hybridized, the total input of counts in the reaction and the percent recovery of potentially hybridizable counts obtained from internal standards, the percentage of newly synthesized nuclear RNA represented by specific gene transcript products was estimated according to the equation

$$\% \text{ RNA} = \left(\frac{\text{cpm hybridized}}{\text{total cpm input}}\right)\left(\frac{\text{total [}^{32}\text{P]cRNA input}}{\text{[}^{32}\text{P]cRNA hybridized}}\right)$$

Under the conditions employed, the calculated values were 0.058%, 0.007%, and 0.002% for ovalbumin, Y, and X gene transcripts, respectively. These values are consistent with the results obtained in the R_0t analysis studies described above and suggest that the different steady-state levels of X, Y, and ovalbumin gene transcripts reflect mostly differential rates of transcription of the three closely related genes.

Although the labeling time was short (7.5 min), it was still possible that differential levels of ovalbumin, Y, and X gene transcripts might be derived from differential rate of turnover. We have thus carried out the pulse–chase experiment by exposing the labeled cells to an excess of unlabeled uridine and cytidine (5 mM each) in order to determine whether the difference can be due to RNA turnover. As shown in Table I, the relative concentration of ovalbumin, Y, and X gene transcripts in the chase samples is strictly proportional to that of the pulse-labeled RNA transcripts. The overall decrease of radioactivity in the RNA transcripts of all three genes is most probably due to splicing of the precursor RNAs and simultaneous degradation of intervening sequence transcripts. These results would indicate that the ovalbumin gene and its pseudogenes are transcribed at different rates in the hormone-stimulated oviduct tissue, and this difference is reflected in their respective concentration levels at steady state.

Discussion

In this paper, we report the isolation and characterization of DNA fragments obtained from a chicken gene library which contain the entire Y and X pseudogenes located 5′ from the authentic ovalbumin gene in the same chromosomal locus of the chick genome. Taken together, the previous report by Royal et al. (1979) and the current study show that all three genes are comprised of eight structural gene segments separated by seven intervening sequences of varying lengths. The similarities in sequence organization and the shared sequence homology between the pseudogenes and the authentic ovalbumin gene would suggest a common evolutionary origin of the genes. The ovalbumin gene–pseudogene locus in chicken is analogous to the globin gene loci in rabbit and human (Flavell et al., 1978; Lawn et al., 1978). The σ and β genes, for example, appear to have arisen by a gene duplication process and are closely linked. Both genes share regions of sequence homology and have similar structural and intervening sequence arrangements. Furthermore, the sequence-homologous regions of the genes appear to mainly reside within the structural segments, suggesting that intervening sequences had diverged more rapidly than the structural sequences. This observation is reminiscent of the α, β major, and β minor globin genes in the mouse (Tiemeier et al., 1978; Konkel et al., 1979; Leder et al., 1978; Nishioka & Leder, 1979).

Both of the X and Y pseudogenes are transcribed in the hormonally stimulated chick oviduct cells. We have demonstrated that specific restriction endonuclease-generated fragments can be prepared from the Y and X genes which share only limited sequence homology with the ovalbumin gene. These fragments are suitable for use as gene-specific probes for studying pseudogene expression in the chick oviduct system. Utilizing these gene-specific hybridization probes, we have investigated the expression of X and Y genes in estrogen-stimulated oviduct RNA preparations by Northern transfer studies. That the mRNA and putative precursor bands detected with the $Y_{1.2}$ probe represent Y gene transcripts and not cross-hybridization of the Y probe with ovalbumin gene transcripts was demonstrated by competition experiments. Hybridization signals obtained from 20 μg of nuclear RNA from stimulated oviduct by using the Y-specific probe were reduced significantly when hybridization reactions were carried out in the presence of 300 μg of unlabeled pY12.4 DNA but were not diminished by a similar excess of pOV12 or pX8.0 DNA. Similarly, specific precursor and mature X RNA sequences were detected in stimulated oviduct nuclear RNA by using the 4.6 probe. The ability to eliminate the hybridization signals by competition with unlabeled Y or X DNA but not with unlabeled ovalbumin DNA is of critical importance due to the abundance of ovalbumin gene transcripts in the stimulated chick oviduct and the presence of sequence homology among the genes. Without the competition studies, it would have been difficult to establish that the signals observed by hybridization were indeed specific Y and X gene transcripts instead of cross-reaction with the ovalbumin gene transcript.

Steady-state levels of X, Y, and ovalbumin gene transcripts in hormonally stimulated and withdrawn oviduct nuclear RNA preparations were determined through the use of the gene-specific hybridization probes under conditions of RNA excess. The results indicate that while transcripts from all three genes are present in low concentrations in withdrawn oviducts (<1 molecule per nucleus), the concentration of ovalbumin gene transcript showed a marked increase in estrogen-stimulated oviduct RNA preparations, while the concentrations of Y and X gene transcripts were enhanced to levels of about 10% and 1% of that of authentic ovalbumin RNA, respectively. The levels of X gene transcripts were so low in the stimulated oviduct that it was previously reported as unresponsive to hormonal stimulation on a qualitative basis (Woo et al., 1979). Since the hybridization probes are mostly composed of intervening sequence, analysis of X and Y steady-state levels in cytoplasmic RNA was not feasible. Thus, the corresponding intervening sequence probes for the authentic ovalbumin gene (pOV1.8 and pOV2.4) were used in these studies for comparison purposes, and our quantitation of ovalbumin, Y, and

X gene transcripts in oviduct nuclear RNA is really a measure of the intracellular concentration of precursor RNA rather than mature mRNA of the respective genes. Depending on the extent of the structural gene regions present in each of the probes used, the relative concentration of the two final mature mRNA levels may vary slightly from the estimated value. The extent of such variations, however, should be minimal since similar results were obtained by using the entire ovalbumin and Y gene DNAs as probes for hybridization. Thus, these results support the concept that there is a graded response in gene transcript accumulation to steroid hormone induction such that ovalbumin > Y > X. The studies utilizing newly synthesized RNA by pulse labeling and chase with unlabeled nucleotides suggest also that the transcription rate for ovalbumin is greater than Y and X in hormonally stimulated oviduct cells.

Although we do not know as yet whether the X and Y genes fulfill a functional role in the developing or mature oviduct tissue, the fact that the member genes of a closely linked gene cluster, existing in a presumably similar environment, are expressed as such different levels in response to steroid hormones is of great interest. While the precise cellular mechanism by which accumulation of pseudogene transcripts occurs remains to be determined, our studies designed to measure their rates of transcription in hormonally stimulated oviduct cells suggest that the steady-state levels of X and Y gene transcripts, like that of ovalbumin, are regulated to a large degree at the level of transcription. This being the case, one may wonder why the transcription rate of the X pseudogene is so poor compared to the authentic ovalbumin gene if in fact it has arisen by a genetic duplication event. Thus, it would be of immediate interest to compare the nucleotide sequence organization at putative regulatory sites located at the regions of initiation of transcription at each of these differentially expressed genes.

Acknowledgments

The authors thank Larry Day, William Tate, Jill Lahti, Sulaf Skory, and Iris Fung for excellent technical assistance and Drs. Jeffrey Nordstrom, Eugene Lai, and Dennis Roop for helpful discussions.

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