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The ovalbumin gene: Structural sequences in native chicken DNA are not contiguous*

(gene splicing/restriction mapping/Southern hybridization/nick-translated recombinant plasmid/R-loop formation)

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ABSTRACT **The sequence organization of the structural ovalbumin gene and flanking sequences in native chicken DNA was stuided by restriction mapping and filter hybridization using a nick-translated probe generated from pOV230, a recombinant plasmid that contains a full-length ovalbumin DNA synthesized from ovalbumin mRNA. The structural sequences of the ovalbumin gene in native chicken DNA were found to be noncontiguous because at least two restriction endonucleases that do not cut the structural sequence do cleave the natural gene into multiple fragments by cleaving within nonstructural sequences interspersed between the structural sequences. The observation that all ovalbumin DNA-containing sequences were contained within a single DNA fragment generated by *Bam*HI digestion of total chicken DNA has allowed us to construct an inclusive restriction map of the natural ovalbumin gene which contains at least two "insert regions." These regions may be further subdivided into alternating structural and insert sequences. Both insert regions were located within the peptide-coding regions of the gene and the sizes of these insert regions were estimated to be approximately 1.0 and 1.5 kilobase pairs, respectively.**

Recent studies have indicated that the regulation, by steroid hormones, of expression of the ovalbumin gene in the chicken oviduct is via a "transcriptional control" mechanism (1–10). To further our understanding of the detailed molecular mechanism of this important regulatory event, large quantities of the natural ovalbumin gene are needed to study its interactions with hormone–receptor complexes, RNA polymerase, and chromosomal components *in vitro*. In order to carry out these studies, the structure and organization of the gene coding for ovalbumin in native chicken DNA must be established so that the gene may subsequently be purified and amplified by molecular cloning. Total chicken DNA was therefore digested exhaustively with various restriction endonucleases and the DNA fragments were resolved by electrophoresis on agarose gels. To detect the DNA fragments containing ovalbumin DNA sequences, the DNA was transferred from the gels to nitrocellulose filters by the method of Southern (11). The nitrocellulose filters were subsequently used for hybridization with specific probes generated from pOV230, a recombinant plasmid containing double-stranded DNA synthesized from full-length ovalbumin cDNA (12). The results of the present study have permitted us to construct a physical map of the natural ovalbumin gene and to conclude that the structural sequences coding for ovalbumin are not contiguous in native chicken DNA.

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MATERIALS AND METHODS

Restriction Digestion of Chicken DNA and Filter Hybridization after Southern Transfer. Chicken DNA, free of protein and RNA, was prepared from chicken embryo, liver, and oviduct epithelial cells by a modification of the procedure of Marmur (13) as described (14). Total chicken DNA (50 μg) was digested in a 100 μl reaction mixture containing 12 units of endonuclease in the appropriate buffer. The reaction mixture was incubated at 37° for 10 hr. The endonuclease digests were heated at 68° for 5 min before electrophoresis on slab agarose gels in Tris·acetate buffer as described (12). The DNA was subsequently transferred onto nitrocellulose filters by the method of Southern (11), and the filters were baked at 68° for 4 hr and then soaked for 16 hr in 0.90 M NaCl/0.09 M sodium citrate containing 0.02% Ficoll, polyvinylpyrolidone, and bovine serum albumin according to the procedure of Denhardt (15). The filters were hybridized with ^{32}P-labeled DNA probes in the same solution containing 0.5% sodium dodecyl sulfate and 1 mM EDTA at 68° for 12 hr, washed three times with 0.30 M NaCl/0.03 M sodium citrate/0.5% sodium dodecyl sulfate at 68° for a total of 6 hr and exposed to x-ray film in the presence of a Dupont Cronex intensifying screen at −20° for up to 3 days.

RESULTS

pOV230 is a chimeric plasmid previously constructed in our laboratory that contains a full-length ovalbumin DNA insert (12). It was digested simultaneously with endonucleases *Hae* III and *Hin*dIII, and the DNA fragments were resolved by electrophoresis in agarose gels. This double enzymatic digestion yielded two fragments containing the entire structural ovalbumin DNA sequences (Fig. 1*A*). The 1.15-kilobase pair (kb)(OV_L) and the 1.45-kb (OV_R) fragments contain DNA sequences corresponding to the 5′ terminus and the 3′ terminus of ovalbumin mRNA with respect to the single *Hae* III cleavage site present in ovalbumin cDNA, respectively (12, 16). The sections of the gel containing the two DNA fragments were excised separately and the DNA was recovered by diffusion

Abbreviations: $dsDNA_{ov}$, double-stranded DNA synthesized from full-length ovalbumin cDNA: kb, kilobase pairs; OV_L, hybridization probe specific to the left half of the structural ovalbumin gene; OV_R, hybridization probe specific to the right half of the structural ovalbumin gene; $cDNA_{ov}$, ovalbumin complementary DNA.

* This manuscript is no. 8 in a series of publications dealing with the structure, organization, function, and regulation of this gene in the chicken oviduct. Paper no. 7 is ref. 14.

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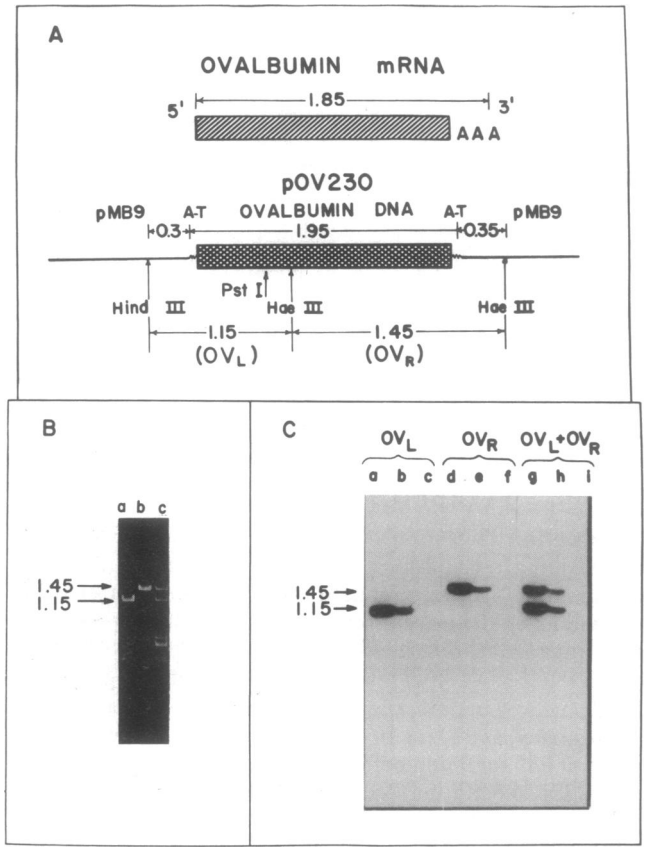

FIG. 1. Separation of OV_L and OV_R. (*A*) Orientation of the full-length $dsDNA_{ov}$ insert in pOV230 (12). □, dsDNA insert; —, pMB9 DNA; ⁓, dA-dT linkers. The sites of cleavage of this recombinant DNA by *Pst* I, *Hue* III, and *Hin*dIII are indicated by the arrows, and the numbers represent the restriction fragment sizes in kilobases. (*B*) A 2% agarose gel of the isolated OV_L and OV_R after staining by ethidium bromide. Lanes: a, 1 μg of OV_L; b, I μg of OV_R; and c, 1 μg of pOV230 cleaved with *Hae* III and *Hin*dIII. (*C*) Radioautogram of a 2% agarose gel containing various amounts of OV_L and OV_R after hybridization with the ^{32}P-labeled nick-translated probes; 10, 1, and 0.1 mg of OV_L and OV_R were applied separately to lanes a, b, and c and d, e, and f and together in lanes g, h, and i, respectively.

(17). Subsequent electrophoretic analysis showed that up to 1 μg each of the DNA fragments migrated as single bands in agarose gels (Fig. 1*B*). The purified left and right DNA fragments were nick-translated with [^{32}P]dGTP to about 8×10^7 cpm/μg by the method of Maniatis *et al.* (18). To test the purity and sensitivity of the radioactive DNA probes, various amounts of pOV230 digested with *Hae* III plus *Hin*dIII were electrophoresed on an agarose gel. The DNA was transferred onto a nitrocellulose filter paper and subsequently allowed to hybridize with the nick-translated DNA preparations followed by extensive washing and radioautography. Both radioactive DNAs hybridized only to the proper DNA bands, with less than 1% cross-contamination (Fig. 1*C*).

Total chicken DNA was digested with *Hae* III, and 15 μg of the DNA was electrophoresed on a 1% agarose gel. Because there is only one *Hae* III site in $dsDNA_{ov}$ (16), a total of two DNA bands was expected with the use of both DNA probes, and only one band was expected when either one of the DNA probes was used. OV_R yielded one DNA band at 2.5 kb whereas OV_L yielded two bands at 1.3 and 1.8 kb (Fig. 2*A*). When both probes were used in the hybridization reaction, all three bands were observed in the presence or absence of a large excess of

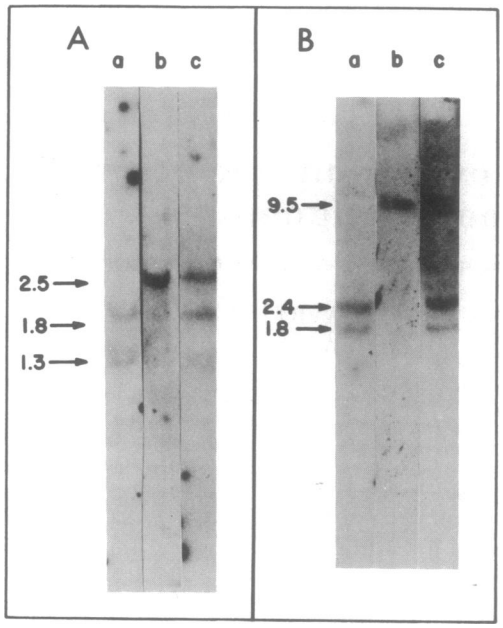

FIG. 2. Radioautograms of total chicken DNA after restriction enzyme digestion and hybridization with the nick-translated probes. Lanes: a, OV_L alone; b, OV_R alone; c, OV_L plus OV_R. (*A*) After *Hae* III digestion. (*B*) After *Eco*RI digestion. Upon prolonged exposure with some DNA fractions, after RPC-5 column chromatography, two additional bands at 3.2 and 1.3 kb were usually observed.

added poly(A). This experiment suggests that there exists a *Hae* III cleavage site in the left half of the structural ovalbumin gene in native chicken DNA that is missing in $dsDNA_{ov}$ (16). When *Eco*RI digested-DNA was electrophoresed on a 1% agarose gel and allowed to hybridize with the specific radioactive probes, a total of three bands was observed (Fig. 2*B*). Because *Eco*RI does not cleave $dsDNA_{ov}$ (16), this experiment suggests that there are two *Eco*RI sites within the natural ovalbumin structural sequence in chicken DNA. Furthermore, when the *Eco*RI digested-DNA was allowed to hybridize with OV_L and OV_R separately, one band approximately 9.5 kb long was generated with OV_R, and two bands 2.4 and 1.8 kb long were generated with OV_L (Fig. 2*B*). Thus, both *Eco*RI cleavage sites seem to be located in the left half of the structural sequence of the natural ovalbumin gene.

To demonstrate further that the observed DNA bands were not due to procedural artifacts and that they contained ovalbumin DNA sequences, we repeated these experiments using *Eco*RI-digested total chicken DNA that was enriched for ovalbumin-containing DNA sequences by R-loop formation with purified ovalbumin mRNA (19, 20) followed by chromatography on an oligo(dt)-cellulose column. A portion of the low-salt column eluate was analyzed for the concentration of ovalbumin DNA sequence by hybridization with ^3H-labeled ovalbumin complementary DNA ([^3H]$cDNA_{ov}$) in liquid medium after all mRNAs were destroyed to mononucleotides by incubation at 37° for 16 hr in the presence of 0.3 M NaOH (Fig. 3*A*). Although total chicken DNA hybridized at very high C_0t values, the DNA after R-loop formation at 43° followed by oligo(dT)-cellulose column chromatography hybridized with $cDNA_{ov}$ at a rate approximately 20 times faster than that of total chicken DNA. When these partially purified *Eco*RI DNA fragments were electrophoresed on agarose gels and allowed to hybridize with the nick-translated pOV230 probes, three bands identical to those found in total chicken DNA were observed (Fig. 3*B*). Furthermore, the intensities of the bands from

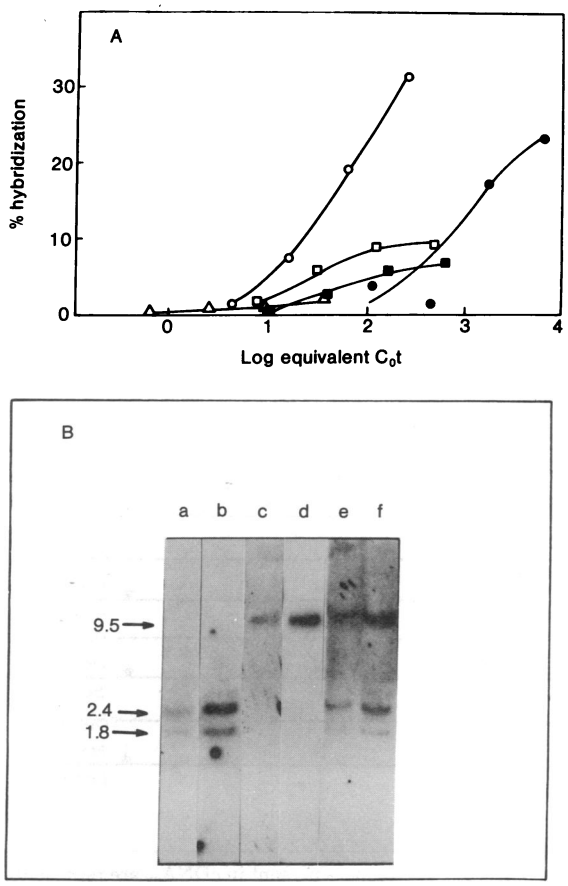

FIG. 3. Enrichment of ovalbumin DNA-containing *Eco*RI fragments by R-loop formation. (*A*) Hybridization curves with [^{3}H]-cDNA$_{ov}$ and DNA fractions recovered from oligo(dT)-cellulose column chromatography after R-loop formation. The hybridization reactions were carried out in 10 mM Tris·HCl, pH 7.0/0.6 M NaCl/2 mM EDTA at 68° and assay for hybrid formation by S1 treatment was performed as described (7). ●, Total *Eco*RI-digested DNA; △, □, ○, and ■, DNA eluted from the oligo(dT)-cellulose column after R-loop formation at 25°, 39°, 43°, and 47°, respectively. (*B*) Radioautograms of a 1% agarose gel: 15 μg of total *Eco*RI-digested chicken DNA and 3 μg of R-loop-enriched DNA were applied to lanes a, c, and e and b, d, and f, respectively. The probes used in the hybridization reactions were OV$_L$ in lanes a and b, OV$_R$ in lanes c and d, and both in lanes e and f.

3 μg of this DNA preparation were approximately 4 times greater than those derived from 15 μg of total *Eco*RI-digested chicken DNA. Because the multiple DNA bands observed in these experiments could be enriched by hybridization with purified ovalbumin mRNA, they must indeed contain at least part of the corresponding structural gene sequence.

To establish a preliminary restriction map of the natural ovalbumin gene within the chicken genome, we first attempted to establish the correct orientation of the three *Eco*RI fragments. Because the 9.5-kb *Eco*RI fragment hybridized only with OV$_R$ whereas the 2.4- and 1.8-kb fragments hybridized only with OV$_L$, the latter DNA fragments must be derived from the left side of the natural ovalbumin gene. When a probe was generated from OV$_L$ after removal of a 0.35-kb fragment by *Pst* I digestion (Fig. 1*A*), only the 2.4-kb fragment was obtained (data not shown). Thus, this fragment must be derived from the left end of the ovalbumin gene, and the four *Eco*RI sites (*Eco*-RI$_{a,b,c,d}$) can be ordered as shown in Fig. 4. Total chicken DNA was then digested with various restriction endonucleases in the presence and absence of *Eco*RI. The DNA digests were again electrophoresed on agarose gels and hybridized with the specific probes. The results are shown in Fig. 5 and sizes of all DNA fragments are summarized in Table 1. *Bam*HI, an enzyme that does not cleave dsDNA$_{ov}$ (16), yielded a single DNA band of about 25 kb with either OV$_L$ or OV$_R$, as expected. Codigestion with *Eco*RI resulted in three bands. The two small bands hybridized only with OV$_L$ and were identical to those generated by *Eco*RI alone. The 9.5-kb *Eco*RI fragment was decreased to a 5.2-kb fragment, indicating that there exists a *Bam*HI cleavage site (*Bam*HI$_b$) 5.2 kb to the right of *Eco*RI$_c$. The other *Bam*HI site (*Bam*HI$_a$) should then be approximately 25 kb to the left of *Bam*HI$_b$ (Fig. 4).

*Hin*dIII, like *Eco*RI, does not cleave dsDNA$_{ov}$ (16). It also produced more than one band from total chicken DNA, indicating the existence of a *Hin*dIII site in native chicken ovalbumin DNA. The sizes of the two DNA bands were 5.0 and 3.5 kb and both bands could be detected when hybridization was carried out with OV$_L$ alone (Fig. 5). Hence, the *Hin*dIII site must be located at the left half of the ovalbumin gene. Because only the 5.0-kb fragment was observed with OV$_R$, there should be a *Hin*dIII site (*Hin*dIII$_c$) located at 5.0 kb to the right of *Hin*dIII$_b$. Furthermore, *Hin*dIII$_c$ should be 2.8 kb to the right of *Eco*RI$_c$ because the 9.5-kb *Eco*RI fragment was decreased to 2.8 kb by *Hin*dIII digestion. Consequently, *Hin*dIII$_b$ is 2.2-kb to the left of *Eco*RI$_c$, and the third *Hin*dIII site (*Hin*dIII$_a$) must then be located 3.5 and 5.7 kb to the left of *Hin*dIII$_b$ and *Eco*-RI$_c$, respectively (Fig. 4).

Although there is a *Pst* I site in the left half of dsDNA$_{ov}$, *Pst* I digestion of chicken DNA resulted in only a 4.7-kb band with both OV$_L$ and OV$_R$ (Fig. 5), suggesting the presence of *Pst* I sites 4.7 kb from both sides of this *Pst* I site (*Pst* I$_c$). Because the 9.5-kb *Eco*RI fragment was digested to 3.0 kb by *Pst* I, there should be a *Pst* I site (*Pst* I$_d$) 3.0 kb to the right of *Eco*RI$_c$. Accordingly, *Pst* I$_c$ should be 1.7 kb to the left of *Eco*RI$_c$. Indeed, the 1.8-kb *Eco*RI fragment detected by OV$_L$ was digested to 1.7 kb by *Pst* I (Fig. 5 and Table 1). Hence, *Eco*RI$_b$ should be 0.1 kb to the left of *Pst* I$_c$. During the double digestion, the 2.4-kb *Eco*RI fragment was unexpectedly cleaved to a 1.9-kb fragment (Table 1), suggesting the presence of an additional *Pst* I site (*Pst* I$_b$) between *Eco*RI$_a$ and *Eco*RI$_b$. This *Pst* I$_b$ site should be 0.5 kb to the left of *Eco*RI$_b$ because positioning this *Pst* I site 0.5 kb to the right of *Eco*RI$_a$ would have produced a 2.0-kb fragment instead of the observed 4.7-kb fragment upon digestion of chicken DNA by *Pst* I alone. The fourth *Pst* I site (*Pst* I$_a$) should then be 4.7 kb to the left of *Pst* I$_b$ (Fig. 4).

DISCUSSION

We have attempted to probe the structure and organization of the structural ovalbumin gene and flanking DNA sequences in genomic chicken DNA by restriction mapping and filter hybridization using probes that were generated from a recombinant plasmid containing a full-length dsDNA$_{ov}$ insert. The fact that these observed DNA bands were enrichable by hybridization with the corresponding mRNA strongly argues against the possibility that all of these observed bands arose from procedural artifacts. Our data are consistent with those obtained recently by Weinstock *et al.* (21), and the fidelity of these experimental procedures has recently been established by Botchan *et al.* (22) to study the integration of simian virus 40 DNA into host DNA and by Jeffreys and Flavell (23), using a nick-translated rabbit β-globin DNA-containing recombinant plasmid, to determine the restriction map of DNA sequences flanking the β-globin gene in native rabbit DNA.

Our results of multiple DNA bands after digestion of total chicken DNA with a restriction enzyme that has no cleavage

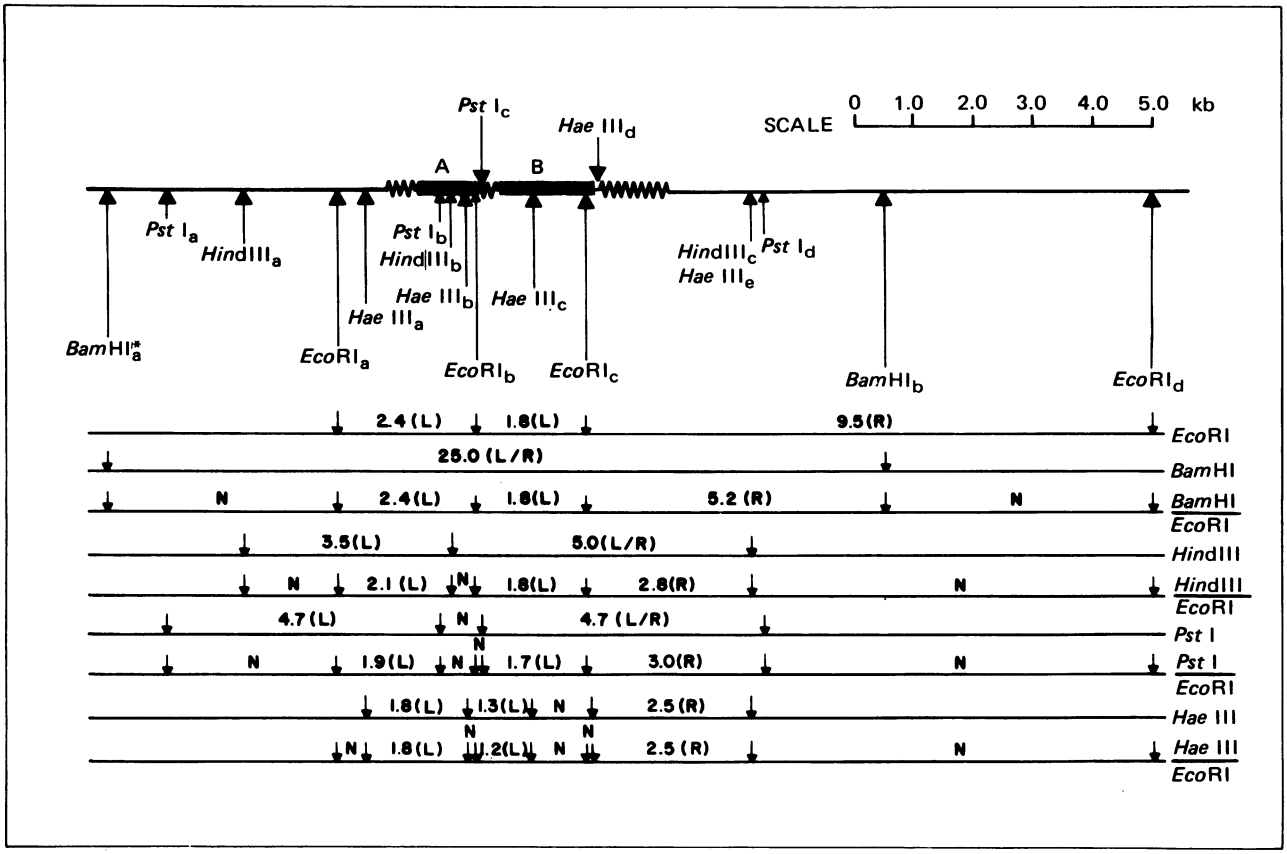

FIG. 4. Model for the organization of the natural ovalbumin gene in chicken DNA. DNA sequences present in $cDNA_{ov}$ are represented by ~, insertion sequences are represented by ■, and flanking DNA sequences are represented by —. Various restriction sites on the DNA are shown by the arrows. Those above the line are restriction sites present in $dsDNA_{ov}$ and those below are predicted by data summarized in Table 1. An exception to the scale is the *Bam*HI$^*_a$ site which should be 25 kb to the left of the *Bam*HI$_b$ site. The generation of DNA fragments of different sizes by various restriction digestions of this DNA is shown below the model: numbers represent the sizes of DNA fragments in kilobases; (L), (R) and (L/R) indicate that the DNA fragment was detected by hybridization with OV_L, OV_R, or both OV_L and OV_R, respectively. N represents DNA fragments that should have resulted from various restriction digestions but were not detected in the radioautograms because there was no DNA sequence complementary to the probes used or the lengths of the complementary sequences were insufficient to form stable hybrids under the conditions used.

site within the structure sequence could be explained in four ways: (*i*) there may be more than one copy of the ovalbumin gene in the chicken genome; (*ii*) there may be heterogeneity in chicken DNA sequences that flank the structural ovalbumin gene; (*iii*) various parts of the structural ovalbumin DNA sequence may be derived from different parts of the chicken genome; and (*iv*) noncoding insertion sequences may be present within the structural ovalbumin gene. However, the probes used in these experiments were specific for the left and right halves of the structural ovalbumin gene (Fig. 1). Therefore, all three *Eco*RI-generated bands should have been obtained when either probe was used alone if the multiple bands were derived either from the presence of multiple ovalbumin gene copies or if there existed heterogeneity in the DNA sequences flanking the structural ovalbumin gene in native chicken DNA. Our data in Fig. 2 clearly demonstrate otherwise, and we are obligated to conclude that the structural ovalbumin gene sequences are

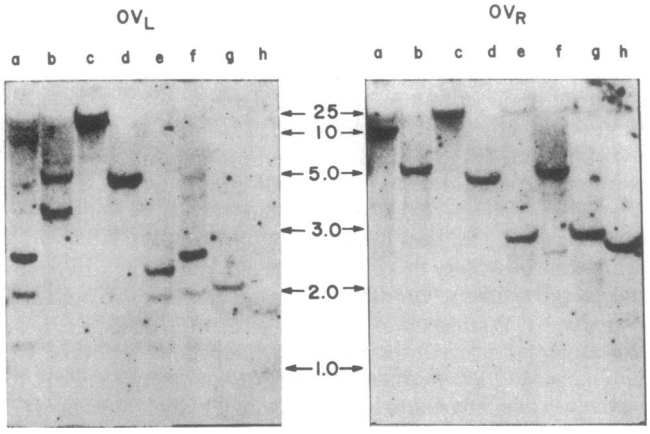

FIG. 5. Radioautograms of 1% agarose gels containing chicken DNA digested with various restriction enzymes. (*Left*) OV_L. (*Right*) OV_R. Lanes: a, *Eco*RI; b; *Hin*dIII; c, *Bam*HI; d, *Pst* I; e, *Eco*RI plus *Hin*dIII; f, *Eco*RI plus *Bam*HI; g, *Eco*RI plus *Pst* I; and h, *Eco*RI plus *Hae* III.

Table 1. Sizes of ovalbumin sequence-containing chicken DNA fragments after restriction enzyme digestion

	Size after digestion, kb					Probe used
	*Eco*RI	*Bam*HI	*Hin*dIII	*Pst* I	*Hae* III	
−*Eco*RI	9.5	25.0	5.0	4.7	2.5	OV_R
+*Eco*RI	9.5	5.2	2.8	3.0	2.5	OV_R
−*Eco*RI	2.4	25.0	5.0	4.7	1.8	OV_L
	1.8		3.5		1.3	
+*Eco*RI	2.4	2.4*	2.1*	1.9*	1.8*	OV_L
	1.8	1.8†	1.8†	1.7†	1.3†	

* These fragments were derived from the 2.4-kb *Eco*RI fragment.
† These fragments were derived from the 1.8-kb *Eco*RI fragment.

not contiguous in native chicken DNA. Furthermore, all ovalbumin DNA-containing fragments are probably not derived from different parts of the chicken genome because only one band was observed after digestion with *Bam*HI. Thus, the multiple bands generated by various restriction enzymes must be due to the presence of respective cleavage sites in the inserts or spacers located within the structural ovalbumin gene.

It has recently been shown that there exist several inserts within the promoter regions of several mRNAs in adenovirus II DNA (24) and one or more inserts in the structural genes coding for rabbit and mouse β-globins and a mouse immunoglobin light chain (25–27). A model for the sequence organization of the ovalbumin gene within native chicken DNA was thus constructed (Fig. 4). In this preliminary model we propose that there are a minimum of two insert regions located within the left half of the structural ovalbumin gene. Insert A is located rather close to the left of the single *Pst* I_c site present in $dsDNA_{ov}$, and it contains at least one cleavage site each for *Pst* I, *Hin*dIII, *Hae* III, and *Eco*RI. Because *Pst* I_b is 0.5 kb to the left of *Eco*RI_b and both sites are contained in this insert, its size must be in excess of 0.5 kb. On the other hand, the entire insert is contained within the 1.9-kb *Hae* III_a/Pst I_c fragment in which 0.5 kb is present in the $dsDNA_{ov}$. Thus, the maximum size of insert A must be smaller than 1.4 kb. In addition, insert B is located to the right of the *Pst* I_c site and very close to the left of the *Hae* III_d site present in $dsDNA_{ov}$ and should contain at least one *Hae* III site and one *Eco*RI site. To accommondate all the fragment sizes generated by *Hae* III, *Pst* I, and *Eco*RI within the 0.25-kb span between *Pst* I_c and *Hae* III_d in $dsDNA_{ov}$, the length of insert B should be approximately 1.5 kb. Finally, we wish to emphasize that inserts A and B are regions of unknown sequences within the structural gene. It is entirely possible that these regions are further subdivided into multiple inserts alternating with structural gene sequences.

At the conclusion of this work, a report by Breathnach *et al.* (28) dealing with the same subject was published. By cleaving a recombinant plasmid constructed in their laboratory and containing 80% of $dsDNA_{ov}$, using *Hha* I, *Pst* I, or *Hin*fI, they generated specific hybridization probes and constructed a restriction map of the ovalbumin gene in native chicken DNA. Although the hybridization probes used in their studies were completely different from ours, a conclusion remarkably similar to ours was reached with regard to the presence of two or more noncoding insert sequences in the natural chicken ovalbumin gene as well as the presence of various restriction sites within the inserts. Our observation that all ovalbumin DNA-containing fragments are contained within one stretch of DNA has enabled us to construct an inclusive map for the natural ovalbumin gene and to estimate the insert lengths. Furthermore, our model (Fig. 4) predicts that codigestion of total chicken DNA with *Pst* I and *Hin*dIII should result in only two DNA fragments, 3.3 and 4.6 kb long. Although we did not carry out a double digestion of chicken DNA with these two enzymes, two DNA fragments, 3.1 and 4.4 kb, were obtained in precisely such an experiment by Breathnach *et al.* (28).

We have recently obtained a preliminary sequence analysis of pOV230 which has demonstrated that within OV_R there are 600–650 nucleotides to the right of the termination codon for ovalbumin synthesis and that within OV_L there are merely about 30 nucleotides to the left of the AUG initiator codon (unpublished data). Because 30 nucleotide pairs are insufficient to form stable hybrids under our hybridization and washing conditions, insert regions A and B must both be located within the peptide-coding sequence of the ovalbumin gene.

It is too early yet to speculate on the function(s) of these insertion sequences within the natural ovalbumin structural gene. With DNA isolated from chicken embryo, liver, and oviduct cells, identical restriction banding patterns were obtained. Because ovalbumin is not synthesized in liver but is produced in great abundance in mature oviduct tubular gland cells, it may be concluded that these inserts do not directly inhibit the expression of the ovalbumin gene in the liver.

Note Added in Proof. While this manuscript was in press, we successfully cloned the *Eco*RI fragments of the ovalbumin gene. Of particular note is that both the 2.4- and 1.8- kb fragments were found to be further subdivided into multiple alternating structural and insert sequences.

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