
The ovalbumin split gene: molecular cloning of Eco RI fragments "c" and "d".

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ABSTRACT

The Eco RI fragments "c" and "d" of the ovalbumin gene (1, 2) have been isolated by molecular cloning. Restriction enzyme mapping and electron microscopy have confirmed that the two fragments contain the same ovalbumin mRNA coding sequences. These sequences are split into two regions which have been mapped in fragments "c" and "d". There is no evidence that the ovalbumin mRNA sequences contained in these fragments could be further interrupted. Our results confirm that the presence of Eco RI fragment "d" in some chickens is due to the existence of an allelic variant of the ovalbumin gene which contains an additional Eco RI site within the region corresponding to Eco RI fragment "c". This additional Eco RI site appears to be the main difference between the two alleles. Finally, our results provide a direct demonstration that most of the ovalbumin mRNA sequences are encoded for by Eco RI fragments "a", "b" and "c".

INTRODUCTION

We have previously demonstrated that the ovalbumin mRNA (ov mRNA) sequences roughly comprised between the Pst I and Hae III restriction enzyme sites of ovalbumin double-stranded cDNA (ov ds-cDNA) (region II of ov-mRNA, see Ref. 1), are encoded for by the Eco RI fragment "c" (1.75 kb) which is located between the Eco RI sites 3 and 2 of the ovalbumin gene (2, 3; see Fig. 1c). It was further shown that the additional presence of fragment "d" (1.25 kb) in Eco RI digests of the DNA of some chickens corresponds to the existence of an allelic variant of the ovalbumin gene containing an additional Eco RI site (Eco 3', see Refs. 2-4 and Fig. 1c). Restriction enzyme mapping of chicken DNA also has revealed that the ov-mRNA coding sequences present in Eco RI fragments "c" and "d" are split at least once and separated by

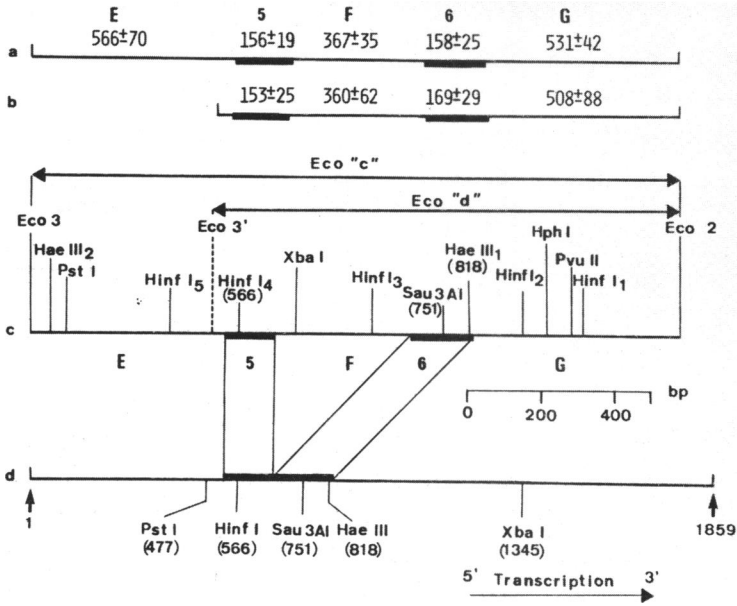


Fig. 1 : Localization of the restriction enzyme sites on the cloned Eco RI fragments "c" and "d" and correlation with a schematic representation of the electron microscopic results and the ovalbumin ds-cDNA restriction map. **a** and **b**) : schematic representation of the electron microscopic results of hybrids between ov-mRNA and cloned fragment "c" (see Fig. 4, A and B) and "d" (see Fig. 4, C and D), respectively. The heavy lines 5 and 6 represent RNA-DNA hybrid regions. E, F and G correspond to single-stranded DNA regions. The numbers correspond to base pairs or nucleotides. **c**) : localization of restriction endonuclease sites on the Eco RI fragments "c" (Eco "c") and "d" (Eco "d"). 5 and 6 represent exons 5 and 6 (see Ref. 9) which can be correlated with the corresponding regions of the ov ds-cDNA [see (d)]. E, F and G correspond to the intervening sequences (introns, see 9) present in fragments "c" and "d". Scale is given in base pairs (bp). **d**) : restriction endonuclease map of ovalbumin ds-cDNA. Only the relevant sites are shown. The locations of the restriction sites are given from the 5' end of ov-mRNA (arrow 1) (9, 12). The arrow 1859 corresponds to the 3' end of ov-mRNA.

an intervening sequence containing an Xba I site which is not present in the corresponding region of ov ds-cDNA (2, 3; see Fig. 1, c and d). To get further insight into the arrangement of this region of the ovalbumin gene, we have cloned the ovalbumin gene fragments "c" and "d" in λ phage. The present analysis of the cloned fragments confirms and extends our previous conclu-

sions drawn from restriction enzyme mapping of the cellular fragments.

MATERIALS AND METHODS

1. Cloning procedure and purification of cloned fragments.

Eco RI fragments "c" and "d" were partially purified by reverse phase chromatography (RPC) as reported previously (2). The isolated fragments were recombined *in vitro* with the purified arms of λ gt WES. λ c (5) and the recombinant DNA was packaged *in vitro* as described previously (6, 7). Plating on *E. coli* and *in situ* hybridization were carried out as described by Garapin et al. (7). After characterization the cloned fragments "c" and "d" were transferred from the phage recombinants into a plasmid vector (pBR 322). The 2.35 kb Eco RI fragment "b" (8) and the 2.6 kb Eco RI-Hind III fragment of the cloned Eco RI fragment "a" containing the entirety of exon 7 (7-9) were also transferred to pBR 322 or to a vector derived from pBR 322 by digestion with Eco RI and Hind III enzymes, respectively.

The cloned fragments were purified on neutral or alkaline sucrose gradients from superhelical DNA plasmids digested with Eco RI (for fragments "b", "c" and "d") or Eco RI + Hind III (for the 2.6 kb Eco RI - Hind III fragment).

2. Electron microscopy.

Hybrids of ov-mRNA and cloned DNA fragments "c" and "d" purified on alkaline sucrose gradients were prepared as described previously (7, 8). Hybrids of ov-mRNA with both Eco RI "b", Eco RI "c" and the 2.6 kb Eco RI - Hind III fragments were obtained in two steps: Eco RI fragment "c" ov-mRNA hybrids were first prepared and then hybridized to Eco RI "b" and Eco RI - Hind III fragments. Heteroduplexes between Eco RI "c" and "d" fragments were prepared as described previously (7, 8). In all cases double-stranded (RF) and single-stranded (SS) ϕ X174 DNAs were used as internal length standards for double- and single-stranded molecules, respectively, assuming that the mass per unit length of a DNA-RNA hybrid and of single-stranded RNA were the same as those of double-stranded DNA and single-stranded DNA, respectively. The samples were spread and the length mea-

surements were carried out as described previously (7, 8).

3. Other procedures and materials.

All other methods and materials were as reported previously (1, 2, 7-9). Biohazards associated with the experiments described in this publication were examined previously by the French National Control Committee, and carried out according to the rules followed by this Committee (Le Progrès Scientifique, N° 191, Nov/Dec 1977), under L3-B2 or L3-B1 conditions.

RESULTS

1. Cloning of the chicken ovalbumin gene fragments "c" and "d".

Eco RI fragments "c" and "d" were partially purified by reverse phase chromatography (RPC) and used for cloning with the vector λ gt WES. λ c (Materials and Methods). Seven independently isolated recombinant phages capable of hybridization to the [32 P]-labelled Hhaov double-stranded cDNA probe ([32 P]-Hhaov probe, see Ref. 1) were obtained from fragment "c" (λ ovEc, 1 to 7), whereas only one such recombinant phage was isolated for fragment "d" (λ ovEd1). Eco RI digests of purified recombinant DNA were separated by electrophoresis on 1% agarose slab gels, examined after ethidium bromide staining and hybridized to the labelled Hhaov ds-cDNA probe. As expected, a 1.25 kb fragment visualized by ethidium bromide staining of the Eco RI digest of clone λ ovEd1 hybridized with the ds-cDNA. Analyses of clones λ ovEc1, λ ovEc2 and λ ovEc3 revealed in all three cases the presence of a stained 1.75 kb fragment, capable of hybridizing to the ds-cDNA, either alone (λ ovEc3) or integrated together with a non-hybridizing fragment of 3 kb (λ ovEc2) or 4.5 kb (λ ovEc1). Since restriction enzyme digestions of the three clones with either Xba I or Hae III enzymes yielded identical hybridizing fragments, only one clone (λ ovEc2) was analyzed further.

2. Restriction enzyme mapping of the cloned Eco RI fragments "c" and "d".

Cloned Eco RI fragments "c" and "d" (Materials and Methods) were digested with restriction enzymes Xba I, Hae III, Hph I,

Table 1 : Mapping of Hae III, Hph I, Pst I, Pvu II, Sau 3AI and Xba I sites within the cloned 1.75 kb fragment "c".

ENZYMES	Hae III	Hph I	Pst I	Pvu II	Sau 3AI	Xba I
Enzyme alone	1140	1410	1670	1470	1130 640	1050 720
Enzyme + Hae III	1140	1140	1100	1140	1070	670 480
Enzyme + Pst I	1100	1310	1670	1370	1030 640	1040 620

The size (in bp) of the fragments hybridizing to the [^{32}P]-labelled ov ds-cDNA probe was determined as described in legend to Fig. 2. Enzyme alone, enzyme + Hae III, enzyme + Pst I refer to the fragments obtained with Hae III, Hph I, Pst I, Pvu II, Sau 3AI, Xba I, alone, or in combination with Hae III or Pst I, respectively.

Pst I, Pvu II and Sau 3AI, either alone or in combination, and the size of the fragments hybridizing with the [^{32}P]-Hhaov probe was determined after agarose gel electrophoresis, as shown in Fig. 2 for Hae III, Hae III/Xba I and Pvu II digests (lanes 1-3 and 15-17 for cloned Eco RI fragments "d" and "c", respectively). Pst I did not cut the Eco RI fragment "d". The results of these size determinations summarized in Tables 1 and 2 allow unambiguous location of the Xba I, Hae III, Hph I, Pst I, Pvu II and Sau 3AI restriction enzyme sites in the 1.75 and 1.25 kb cloned fragments as indicated in Fig. 1c. The Hinf I sites were mapped using the method of Smith and Birnstiel (10). Eco RI fragment "c" was end-labelled with [^{32}P] and cut with Pst I or Pvu II. The larger Eco RI/Pst I or Eco RI/Pvu II fragments were purified by acrylamide gel electrophoresis and then partially digested with Hinf I. The size of the various labelled fragments was determined by autoradiography after agarose gel electrophoresis. The two sets of results (not shown) were in excellent agreement and allow the unambiguous location of 5 Hinf I sites at about 260, 430, 840, 1200 and 1390 bp from Eco RI site 2 (Fig. 1c). The first four sites, Hinf I₁ to Hinf I₄, were also

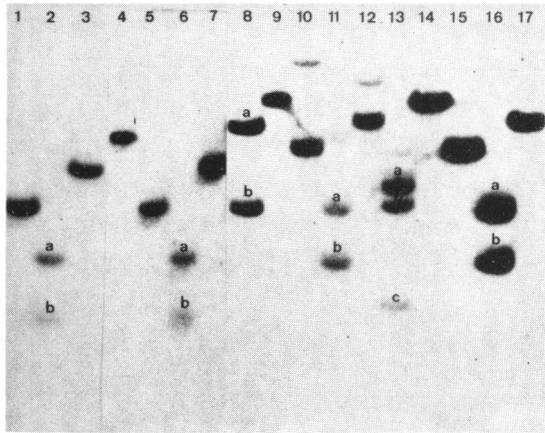


Fig. 2 : Restriction enzyme mapping of cloned Eco RI fragments "c" and "d" and the corresponding chromosomal fragments. Cloned fragments or chromosomal Eco RI fragments separated and partially purified by reverse phase chromatography (RPC-5) were digested with Hae III, Hae III/Xba I or Pvu II endonucleases. The fragments were electrophoresed on 2% agarose slab gels, transferred to nitrocellulose filters, hybridized to the ov ds-cDNA [³²P]-Hhaov probe, and the hybridizing fragments revealed by autoradiography (1). Lanes 8 and 13 : internal markers prepared from pCR1 ov2.1 DNA (11) by cleavage with Hae III (lane 8) and Hinf I (lane 13) restriction endonucleases. The sizes of the hybridizing fragments are (in bp) : lane 8 : a) 1410, b) 720; lane 13 : a) 880, b) 750, c) 300 plus 270. Lanes 1, 2, 3, Hae III, Hae III/Xba I and Pvu II digests of cloned fragment "d". Lanes 5, 6 and 7, Hae III, Hae III/Xba I and Pvu II digests of RPC-purified Eco RI fragment "d" (lane 4). Lanes 10, 11, 12, Hae III, Hae III/Xba I and Pvu II digests of RPC-purified Eco RI fragment "c" (lane 9). Lanes 15, 16, 17, Hae III, Hae III/Xba I and Pvu II digests of cloned fragment "c" (lane 14). The size of the hybridizing fragments are (in bp) : lane 1, 710; lane 2 : a) 470, b) 230; lane 3 : 975; lane 4 : 1270; lane 5 : 710; lane 6 : a) 470, b) 230; lane 7 : 975; lane 9 : 1780; lane 10 : 1140; lane 11 : a) 660, b) 470; lane 12 : 1480; lane 14 : 1780; lane 15 : 1140; lane 16: a) 660, b) 470; lane 17 : 1480.

found to be similarly located with respect to Eco RI site 2 in the cloned fragment "d". Restriction endonucleases Ava I, Bgl I, Bgl II, Hae II, Hha I, Hpa I, Hpa II, Sac I, Sal I, Taq I, Xho I, Bam HI, Hind III and Kpn I did not cleave the cloned Eco RI fragments "c" and "d". The sites for Alu I and Mbo II which cut both fragments very frequently were not mapped.

The above results strongly suggest that Eco RI fragment "d"

Table 2 : Mapping of Hae III, Hph I, Pvu II, Sau 3AI and Xba I sites within the cloned 1.25 kb fragment "d".

ENZYMES	Hae III	Hph I	Pvu II	Sau 3AI	Xba I
Enzyme alone	700	910	980	630	1050 230
Enzyme + Hae III	700	700	700	630	470 230
Enzyme + Xba I	470 230	680 230	750 230	630 400 230	1050 230

The size (in bp) of the fragments hybridizing to the [³²P]-labelled ov ds-cDNA probe was determined as described in legend to Fig. 2. Enzyme alone, enzyme + Hae III, enzyme + Xba I, refer to the fragments obtained with Hae III, Hph I, Pvu II, Sau 3AI, Xba I, alone, or in combination with Hae III or Xba I, respectively.

is contained in its entirety within fragment "c" (Fig. 1c). This was further demonstrated by electron microscopic studies.

3. *Electron microscopic evidence that the cloned Eco RI fragments "c" and "d" share common mRNA-coding and intervening sequences.*

Heteroduplexes were formed between the cloned 1.75 and 1.25 kb fragments. Two such heteroduplex molecules are shown in Fig. 3B. In agreement with the restriction enzyme mapping results, both molecules show an uninterrupted double-stranded segment of about 1300 bp continued by a single-stranded segment of about 500 nucleotides (measurements carried out on 46 heteroduplex molecules gave values of 1280 ± 83 bp for the double-stranded segment). In addition, nuclease S1 treatment (11) of heteroduplex molecules yielded only molecules of 1250 nucleotides after alkaline gel electrophoresis (not shown) indicating that there is very little, if any, mismatching in the double-stranded region of the heteroduplexes.

The location of the ov-mRNA coding (exons) and of the intervening (introns) sequences in the cloned Eco RI fragment "c" was

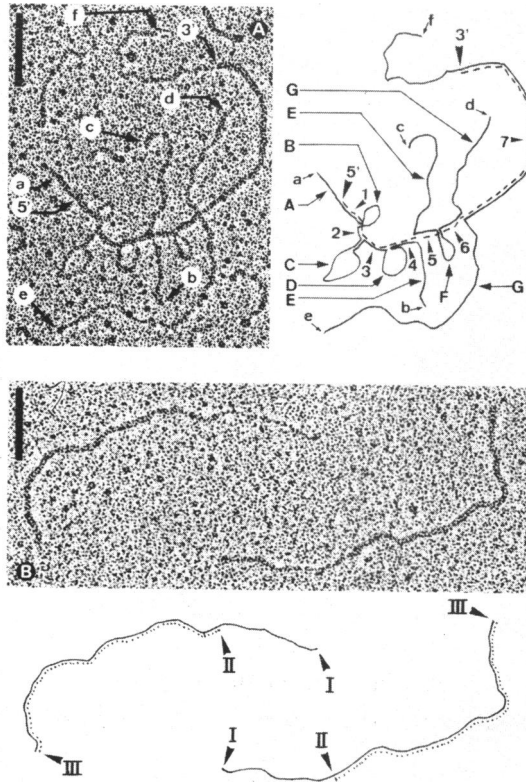


Fig. 3 : A) Electron micrograph of a hybrid between ovalbumin mRNA and the cloned ovalbumin gene Eco RI fragments with a line drawing interpreting the micrograph. Purified ov-mRNA and the cloned 2.35 kb Eco RI fragment "b", the cloned 1.75 kb Eco RI fragment "c" and the 2.6 kb Eco RI-Hind III fragment of the cloned Eco RI fragment "a" were hybridized as described under Material and Methods. In the line drawing, ov-mRNA is represented by a dashed line, whereas the solid line corresponds to single-stranded DNA. 5' and 3' arrows : 5' and 3' ends of ov-mRNA, respectively. a and b, c and d, e and f arrows correspond to the 5' and 3' extremities (in the direction of transcription) of Eco RI fragments "b" and "c", and of the Eco RI-Hind III fragment, respectively. 1 to 7 (the thicker DNA-RNA hybrid regions) correspond to the ovalbumin gene exons, whereas A to G correspond to the 7 introns as previously described and defined by Mandel et al. (2) and by Breathnach et al. (9).

B) Electron micrograph of two heteroduplex molecules between the cloned Eco RI fragments "c" and "d", with line drawings interpreting the micrographs. Arrows I and III, and arrows II and III indicate the ends of the Eco RI fragment "c" and "d", respectively. The dotted line corresponds to one strand of Eco RI fragment "d", whereas the solid line represents one strand of Eco RI fragment "c".

determined by hybridizing this fragment to ov-mRNA in the presence of formamide under conditions where the formation of RNA-DNA hybrids is favoured. Typical hybrid molecules are shown in Fig. 4, A and B. Four single-stranded arms are joined by a central double-stranded RNA-DNA hybrid region from which a single-stranded loop emerges (34 hybrid molecules were measured). The largest single-stranded arm (1047 ± 87 nucleotides) can be digested by RNase (not shown) and corresponds to the 3' moiety of the ov-mRNA which is coded for by Eco RI fragment "a" (1, 2, 7; see Figs. 1d and 3A). The shortest single-stranded arm (465 ± 37 nucleotides) can also be digested by RNase and corresponds therefore to the 5' part of the ov-mRNA which is coded for by Eco RI fragment "b" (1, 2, 8; see also Figs. 1d and 3A). The hybrid molecules may thus be oriented with respect to transcription and the single-stranded DNA arms E and G, the single-stranded DNA loop F and hybrid DNA-RNA segments 5 and 6 (see the line drawings of Fig. 4, A and B), with lengths of 566 ± 70 , 531 ± 42 , 367 ± 35 , 156 ± 19 and 158 ± 25 nucleotides or bp, respectively, may be defined.

When the cloned fragment "d" was hybridized to ov-mRNA very similar hybrid molecules were obtained, but single-stranded DNA arm "E" was missing (compare Fig. 4A and B with Fig. 4C and D). Lengths of 153 ± 25 , 360 ± 62 , 169 ± 29 and 508 ± 88 were measured for segments 5, F, 6 and G (see line drawings of Fig. 4C and D), respectively, in very good agreement with the lengths of the corresponding segments of the hybrid molecules obtained with the cloned fragment "c". A schematic representation of the electron microscopic results is given in Fig. 1, a and b. There is excellent agreement between the lengths of exons 5 and 6 as determined by electron microscopic measurements and their actual lengths as determined by DNA sequencing of the cloned fragments (140-144 and 152-158 bp, respectively; see Ref. 9).

The exons 5 and 6 may be positioned on the restriction maps of fragments "c" and "d" from the above results. When this is done, they are found to surround the sites Hinf I₄ and Sau AI. These sites therefore correspond to the Hinf I and Sau 3AI sites in the ds-cDNA, known from our previous results (2) to map within fragments "c" and "d" and located on ovalbumin ds-cDNA at

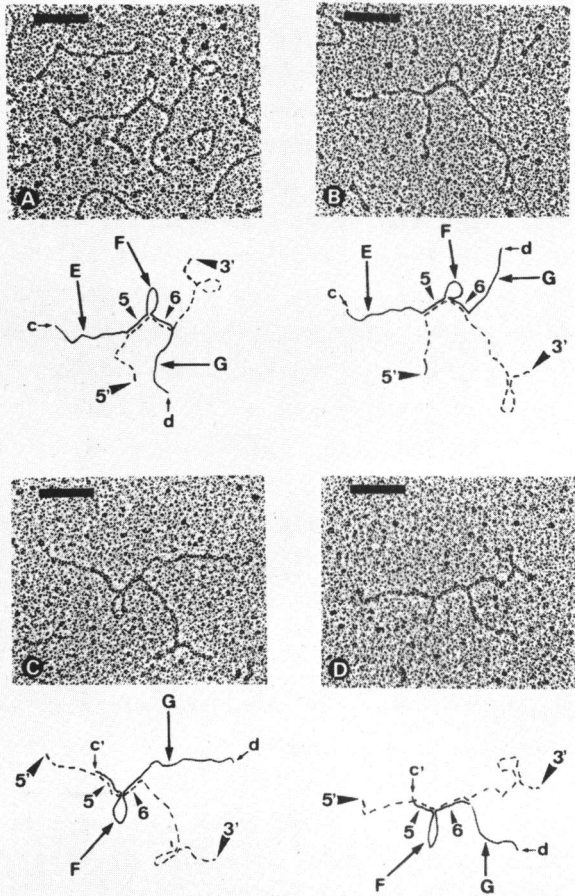


Fig. 4 : Electron micrograph of hybrid molecules between ovalbumin mRNA and the cloned Eco RI fragments "c" and "d". A) and B) : Hybrids between Eco RI fragment "c" and ov-mRNA and line drawings interpreting the micrographs. Fragment "c" extends between arrows c and d (solid lines on the line drawings). 5' and 3' arrows correspond to the 5' and 3' ends of mRNA (dashed line in the line drawings), respectively. The lengths of the two thicker RNA-DNA hybrid regions 5 and 6 are 156 ± 19 and 158 ± 25 bp, respectively. The lengths of the single-stranded DNA regions E, F and G are 566 ± 70 , 367 ± 35 and 531 ± 42 nucleotides, respectively. C) and D) : Hybrids between Eco RI fragment "d" and ov-mRNA and line drawings interpreting the micrographs. Fragment "d" extends between arrows c' and d (solid lines on the line drawings). 5' and 3' arrows are as defined above. The lengths of the thicker RNA-DNA hybrid regions 5 and 6 are 153 ± 25 and 169 ± 29 bp, respectively. The lengths of the single-stranded DNA regions F and G are 360 ± 62 and 508 ± 88 nucleotides, respectively.

positions 566 and 751 (9,12; see Fig. 1d). Since it is known from DNA sequencing (9) that exons 5 and 6 code for ov-mRNA nucleotides comprised between positions 530-531 and 671-673, and between positions 671-673 and 825-828, respectively, the limits of exons 5 and 6 can be drawn as shown in Fig. 1c.

4. *Evidence that the cloned Eco RI fragments "c" and "d" are faithful copies of the corresponding cellular fragments.*

The general organization of the cloned fragments "c" and "d" is similar to that previously reported for their cellular counterparts, since they consist of two exons separated by an intron containing an Xba I site (2,3). In addition, the sizes of the Xba I, Pst I and the Hae III fragments, which hybridize with the [³²P]-labelled Hhaov ds-cDNA probe are very similar for the cloned and the cellular fragments (compare the data of Tables 1 and 2 with the results given in Ref. 2). Further to ascertain that no recombination, deletion or translocation event had occurred during the cloning process, the cloned and the RPC-purified fragments "c" and "d" were digested with a variety of restriction enzymes and the patterns of hybridization with the [³²P]-Hhaov probe compared after separation of the restriction enzyme fragments by agarose gel electrophoresis. It is clear from the results shown in Fig. 2, that digestion with Hae III, Pvu II or Hae III plus Xba I gave identical patterns of hybridization for the cellular and the cloned fragments (compare lanes 5-7 to lanes 1-3 for the cellular and cloned fragment "d", and lanes 10-12 to 15-17 for the cellular and cloned fragment "c"). In addition, the patterns of hybridization after digestion with Mbo II restriction enzyme were the same when the cloned fragments were compared with their cellular counterpart. All of these results indicate that our cloning procedure is reliable, a conclusion which is further supported by the identity between the cloned fragment "d" and its counterpart sequences in the cloned fragment "c" (see above).

5. *Further evidence that the Eco RI fragments "c" and "d" belong to two different alleles of the ovalbumin gene.*

It has been previously proposed (2-4) that the presence of

an additional Eco RI site (Eco 3', see Fig. 1c) in one allelic variant of the ovalbumin gene could be responsible for the presence of either fragment "c" or "d" in the DNA of homozygote chickens (chicken "c" and "d", respectively), and of both fragments in the DNA of heterozygotes. This suggestion can be verified by analyzing with a [³²P]-labelled cloned fragment "c" probe the restriction enzyme patterns obtained from the DNA of the two types of homozygote chickens. For instance, after Eco RI digestion, one would expect to reveal by autoradiography a 1.75 kb fragment in the DNA digest of chickens "c", whereas two fragments, 1.25 and 0.5 kb in length, should be found in the DNA digests of chickens "d". On the other hand, digestion of DNA of both types of chickens with Xba I plus Hae III, or with Xba I plus Pst I, should yield the same hybridizing fragments. These predictions were fulfilled as shown in Fig. 5. The Eco RI digest of DNA of chicken "d" contained two hybridizing fragments of 1.25 and 0.5 kb (lane 1), whereas only one fragment of 1.75 kb was present in the DNA digest of chickens "c" (lane 4). However, the

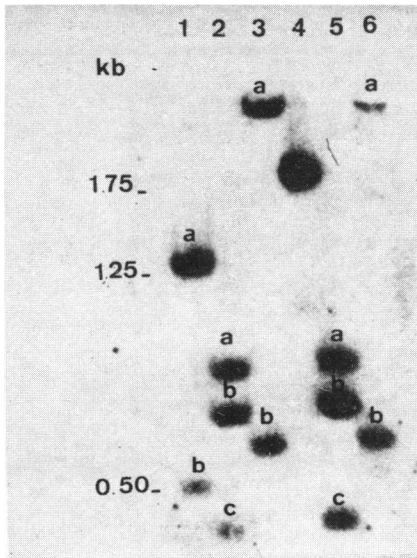


Fig. 5 : Comparison of restriction enzyme fragments produced from chromosomal DNA of chicken "c" or "d" upon digestion with Eco RI, Xba I, Hae III and Pst I. DNAs of two chickens containing either Eco RI fragment "c" (chicken "c", lanes 1-3) or "d" (chicken "d", lanes 4-6) were cut by Eco RI (lanes 1 and 4), Xba I/Hae III (lanes 2 and 5) or Xba I/Pst I (lanes 3 and 6). The resulting fragments were separated on a 2% agarose slab gel, hybridized to the cloned Eco RI fragment "c" labelled by nick-translation with [³²P] and the hybridizing fragments were revealed by autoradiography (the bottom part of the gel only is shown). The sizes of the hybridizing fragments are (in bp) : lane 1 : (a) 1270, (b) 490; lane 2 : (a) 860, (b) 670, (c) 470; lane 3 : (a) 2490, (b) 620; lane 4 : 1760; lane 5 : (a) 860, (b) 670, (c) 470; lane 6 : (a) 2490, (b) 620.

Xba I/Hae III fragments of chickens "d" (lane 2) and "c" (lane 5) were undistinguishable, as well as the Xba I/Pst I fragments (lanes 3 and 6 for chickens "d" and "c", respectively). The lengths of these fragments were as expected from the present and previously reported restriction enzyme maps (2). The fragments containing the Eco 3 site (Fig. 1c) were not detected in these experiments for both the Xba I/Hae III and Xba I/Pst I digests because of the small number of nucleotides able to hybridize to the labelled Eco RI fragment "c" probe. All of these results indicate that the presence of an additional Eco RI site in the DNA of chickens "d" is not linked to any major modifications in the region lying between this Eco RI site and the Eco 3 site (Fig. 1c).

6. *Electron microscopic evidence that most of the ovalbumin mRNA sequences are encoded for by Eco RI fragments "a", "b" and "c".*

The availability of cloned Eco RI fragments "a" (7), "b" (8) and "c" has enabled us to show directly that most of the ovalbumin mRNA sequences are encoded for by these three DNA fragments. Fig. 3A shows an electron micrograph of a hybrid molecule between ov-mRNA and the cloned Eco RI fragments "b", "c" and the Eco RI/Hind III fragment which contains the ov-mRNA coding sequences present in Eco RI fragment "a" (1, 2, 7). In agreement with all of our previous studies on cellular or cloned ovalbumin gene fragments (1, 2, 3, 7, 8, 13) and the schematic representation of the ovalbumin gene given by Breathnach et al. (9), the ov-mRNA sequences appear to be engaged in seven RNA-DNA hybrid regions (which correspond to the ovalbumin exons, 1 to 7 on the line drawing), separated by six single-stranded DNA regions (which correspond to the introns, B to G on the line drawing).

DISCUSSION

The present studies establish that the occurrence of Eco RI fragment "d" in Eco RI digests of the DNA of some chickens corresponds to an allelic variation of the ovalbumin gene which does not affect the ov-mRNA coding sequences. DNA sequencing (9) has

demonstrated unequivocally that the additional Eco RI site (Eco 3') present in the DNA of some chickens is located in intron E, and not in ov-mRNA coding sequences. Such an intronic variation cannot be detected by analyses of the mRNA or the protein and corresponds therefore to a new type of genetic allele. Another possible intronic allelic variation concerning an Hae III site has been previously found in the ovalbumin gene Eco RI fragment "b" (8). How frequent such intronic allelic variations are is presently unknown and will be revealed only by further detailed sequence analysis of cloned introns. In any case it is clear from our present results that the presence of the additional Eco RI site, Eco 3', is not accompanied by any major modifications in the regions of intron E (see Ref. 9) comprised between this Eco RI site and the Eco 3 site (Fig. 1c). It is therefore possible that the presence of the additional Eco 3' site corresponds to a single base pair change similar to those which have been found in the mRNA coding sequences when comparing the ov ds-cDNA sequences obtained by Breathnach et al. (9) and by Mc Reynolds et al. (12).

We have previously concluded from restriction enzyme mapping studies of the chromosomal DNA (2, 3) that the ov-mRNA coding sequences contained in Eco RI fragments "c" and "d" are split into two exons. Our present electron microscopic results fully support this conclusion and do not provide any evidence that the two ov-mRNA coding regions could be further interrupted. In fact, such a possibility has been recently definitely ruled out by sequencing studies (9) which have also revealed the presence of a previously undetected Hae III site located at 16 bp on the right of Hae III₁ site (see Fig. 1c and Fig. 2c of Ref. 9).

Taken together with our previous results (2, 3, 7, 8, 13) our present electron microscopic studies of hybrids between ov-mRNA and the cloned Eco RI fragments establish definitely that all of the ov-mRNA coding sequences present in fragments "a", "b" and "c" are arranged in the cellular DNA in the same order and in the same orientation as in the ovalbumin ds-cDNA. Moreover, the electron microscopic examination of hybrid molecules between ov-mRNA Eco RI fragments "a", "b" and "c" (Fig. 3A) suggests that most, if not all, of the ov-mRNA sequences are en-

coded for by these three DNA fragments in the form of 7 exons separated by 6 introns. That the ov-mRNA coding sequences which are present in exons 1 to 7 correspond, as suggested by the electron micrograph, to sequences which are in direct contiguity in the mRNA, was recently definitely established by DNA sequencing studies (9). However, and in contrast to what was expected from examination of the electron micrograph, the same sequence studies have shown that the first 45 nucleotides at the 5' end of ov-mRNA are not encoded for by Eco RI fragment "b". This observation illustrates well the limit of electron microscopy in detecting regions of non-complementarity.

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