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The presence of ovalbumin mRNA coding sequences in multiple restriction fragments of chicken DNA

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ABSTRACT

Chicken DNA has been digested with restriction enzymes and the size distribution of the DNA fragments containing ovalbumin specific sequences has been examined after separation of the fragments on agarose gels and transfer to nitrocellulose sheets. Hybridisation with terminally ^{32}P-labelled ovalbumin mRNA fragments or with RNA populations transcribed from the DNA of a hybrid plasmid containing ovalbumin sequences was used to locate the DNA fragments coding for ovalbumin. Digestion with enzymes which do not cut within the portion of the ovalbumin gene synthesised from ovalbumin messenger RNA *in vitro* has shown the presence of several defined fragments carrying ovalbumin specific sequences. Possible explanations of these observations are discussed.

INTRODUCTION

There is currently great interest in applying the techniques of genetic manipulation to the analysis of the structure of eukaryotic genomes and to studies on the control of gene action. To date two procedures have been used to derive the DNA sequences to be cloned. In the first procedure double stranded DNA has been synthesised from purified messenger RNA (mRNA) using reverse transcriptase (1-3). One major drawback to this method however is that even full length transcripts will only generate that portion of the transcribed region of the gene which survives post-transcriptional processing. Since some of the major control sequences are likely to be located in non-transcribed regions these cannot be cloned by the reverse transcription method.

The alternative approach avoids this difficulty but requires the isolation of a DNA fragment containing the required gene from total genomic DNA. Due to the complexity of the eukaryotic genome DNA considerable gene enrichment is necessary to make the task of screening recombinant clones an acceptable one. The hybridisation of mRNA or cDNA to genomic DNA fragments has been used to achieve this (4-6). We are currently examining a method for enrichment of the ovalbumin gene in which DNA is digested with a suitable restriction enzyme to give a population of specific fragments which can be separated by gel electrophoresis. By choosing enzymes which do not cut the structural portion of the ovalbumin gene we hoped to generate DNA fragments containing the whole ovalbumin gene plus sequences on either side of the gene and, by using the enzymes sequentially, to facilitate purification. Our initial experiments have produced the unexpected result that total genome digests contain several distinct ovalbumin specific fragments. Different enzymes produce different arrays of ovalbumin specific fragments.

METHODS

Materials

Radioactive nucleotides were obtained from the Radiochemical Centre, Amersham. Nitrocellulose sheets (Sartorius Membrane Filters, 0.45 μ pore size) were obtained from V.A. Howe. Restriction enzymes were purchased initially from Miles Laboratories; larger quantities of some enzymes were isolated as follows: Eco RI by the method of Yoshimori (7) and Hind III by the method of Smith & Wilcox (8). E.coli RNA polymerase was obtained from Boehringer.

Isolation of DNA

DNA was isolated from livers or oviducts of laying hens by a method derived from the methods of Kirby and Cook (9). Tissues

from fasted laying hens were homogenised in 5 volumes of saline citrate at 4°C and poured into 5 volumes of 1% Lithium dodecyl sulphate. The viscous solution was extracted with phenol, the aqueous layer made 3% in sodium chloride and extracted with phenol:chloroform (1:1) and the DNA precipitated with the minimum amount of ethanol. The ethanol was decanted, the DNA dissolved in 0.1 M sodium acetate pH 6 and solid sodium chloride added to 3 M. After standing overnight at 4°C the precipitated RNA was spun down and the supernatant again precipitated with the minimum amount of ethanol and the precipitated DNA dissolved in 0.1 M sodium acetate pH 6. The solution was treated with RNAase A (heat treated to inactivate DNAase, 10 μg/ml) for 1 hr. at 37°C and extracted with phenol:chloroform (1:1). Residual glycogen was removed by partitioning in 2.5 M potassium phosphate : 2-methoxyethanol (10). The DNA solution was again precipitated with ethanol, dissolved in 0.1 M sodium acetate and dialysed exhaustively against 5 mM sodium chloride, in which it was stored at -20°C. The molecular weight of the final product was estimated by agarose gel electrophoresis to be 30 - 40 x 10^6 daltons.

Preparation of ^{32}P labelled mRNA fragments

Ovalbumin mRNA was purified from the oviducts of laying hens as described previously (11). The mRNA (2 μg) was fragmented by digestion in 0.1 M sodium hydroxide for 1 hr. in ice then neutralised and precipitated with ethanol. The fragments were terminally labelled by incubation for 1 hr. at 37°C in a mixture (50 μl) containing 50 μCi of γ^{32}P.ATP, 2 units of polynucleotide kinase, 10 mM β-mercaptoethanol, 5 mM magnesium chloride, 50 mM tris pH 7.6. After this time the reaction mixture was made 0.1% in SDS and passed down a 0.7 x 30 cm column of Sephadex G-50 (med) and the excluded peak precipitated with ethanol. Specific activity of the product was of

the order of 10^7 - 10^8 cpm/μg.

Synthesis of ovalbumin genes in vitro

Full-length single-strand ovalbumin cDNA was prepared essentially as described previously (12) and a poly(dT) tail (average of 40 residues per molecule) was then added to the 3'-terminus using terminal deoxynucleotidyltransferase (P-L Biochemicals). Double-strand cDNA was prepared by first pre-incubating 0.75 μg of T-tailed cDNA at 25°C for 30 minutes in 60 μl containing 2.25 μg oligo$(dA)_{10}$ and 0.2 M KCl, and then further incubating at 37°C for 6 hours in a final volume of 600 μl now containing the following; 0.01% (v/v) Triton X-100, 60 mM KCl, 4 mM $MgCl_2$, 0.1 M Tris-HCl pH 8.3, 5 mM DTT, 0.025 mM dCTP, 0.2 mM dATP, dGTP, and TTP, 250 μCi of α-[^{32}P]dCTP, reverse transcriptase at 45 units per ml, T-tailed cDNA and oligo$(dA)_{10}$ primer. The purified DNA products were then electrophoresed through a 1.4% agarose gel and full-length double-strand ovalbumin cDNA molecules eluted.

Preparation of cRNA from plasmid DNA

Closed circular DNA of the plasmid pCR1.OV2.1(3) was grown from a sample kindly provided by Dr. P. Humphries and Prof. P. Chambon, and was digested with Hpa II to convert it to linear molecules. These fragments or similar fragments from the parent plasmid pCR1 were transcribed using RNA polymerase from E.coli. A typical reaction mixture contained (in 100 μl) plasmid DNA (1 μg), 20 mM tris HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM DTT, 40 mM KCl, 0.1 mM EDTA, 5% glycerol, 150 μCi α-^{32}P-UTP (250 Ci/mMole), 2 μM cold UTP, 100 μM each of ATP, GTP and CTP and 5 units of RNA polymerase. The mixture was incubated for 90 minutes at 37°C then 4 μg of DNAse I was added and incubated a further 15 minutes at 37°C. The mixture was then extracted twice with an equal volume of phenol:chloroform (1:1) and the aqueous layer separated on a small Sephadex G-50 (med) column

in 50 mM sodium chloride, 0.1% SDS. The excluded peak was pooled and precipitated at -20°C with 2 volumes of ethanol. Material prepared by this method has a specific activity of about 5 x 10^7 cpm/μg.

Restriction Enzyme Digestion of DNA

Restriction digests were performed at 37°C using amounts of enzyme calculated to be a 2 fold excess over the amount required for complete digestion. Normal digestion time was 16 hrs.

Agarose Gel Electrophoresis

Agarose slab gels (usually 1%) were prepared and run as described by Sharp et al. (13). The gels, measuring 14 cm x 12 cm x 0.5 cm were run at a constant 50 mA until a bromophenol blue dye marker reached the bottom of the gel.

Nitrocellulose filter methodology

Alkali denaturation of agarose gels and transfer of DNA to nitrocellulose sheets was performed as described by Southern (14). The filters were hybridised to the labelled probes (about 5 x 10^7 cpm) in 6 x SSC for 24 hrs. at 68°C. Hybridisation to mRNA derived probes was conducted in the presence of a large excess (about 5 mg/ml) of non-radioactive chick liver ribosomal RNA. The dried filters were autoradiographed at -70°C using Kodak RP54 X-ray film in a Kodak X-omatic C-1 cassette with regular intensifying screens.

RESULTS AND DISCUSSION

It has been estimated that there is only a single copy of the ovalbumin gene in the chicken haploid genome (15, 16). Evidence has been presented to show that double stranded DNA transcribed from ovalbumin mRNA is not cut by Eco RI or Hind III (17). We have confirmed this finding using double stranded DNA transcribed from ovalbumin mRNA. This material corresponds to the full length of the messenger (18). This material is

unaffected by digestion with Hind III (fig. IA) and Eco RI (fig. IB) but is digested with Hae III (fig. IC). This being so we expected that digestion with Hind III or Eco RI would produce only one band hybridising to an ovalbumin probe after separation on an agarose gel and transfer to nitrocellulose. Our initial experiments which involved hybridising Hind III digested DNA to ^{32}P-labelled ovalbumin mRNA fragments showed at least 3 bands (fig. 2).

By comparison with Hind III digested PM2 DNA molecular weight markers (19) the sizes of the three major bands were estimated to be 4400, 3050 and 2050 base pairs. There are several possible explanations for this result, some of which we have examined. Firstly, it was possible that the standard hybridisation conditions were insufficiently stringent and that imperfect hybridisation to other "ovalbumin-like" sequences was being observed. We therefore repeated the experiment with the additional step of washing the filters in low salt (0.03 x SSC at 65^{o}C) after hybridisation to disaggregate imperfect hybrids. The autoradiograph of a filter so treated was identical with one treated in the normal way.

A second possibility is that the restriction enzyme treatment was incomplete leading to a series of characteristic partial products. This was ruled out by repeating the experiment with larger amounts of enzyme (5 fold) and longer digestion times (24 hrs.). The hybridisation patterns were not changed by these more vigorous treatments.

A third possibility is that the mRNA preparation used to prepare the ^{32}P-labelled probe may have contained contaminating sequences such as ribosomal RNA or other mRNAs. To overcome this difficulty we prepared a labelled probe from a plasmid containing sequences corresponding to 85% of the ovalbumin structural gene (3). Hybridisation of this to Hind III digested

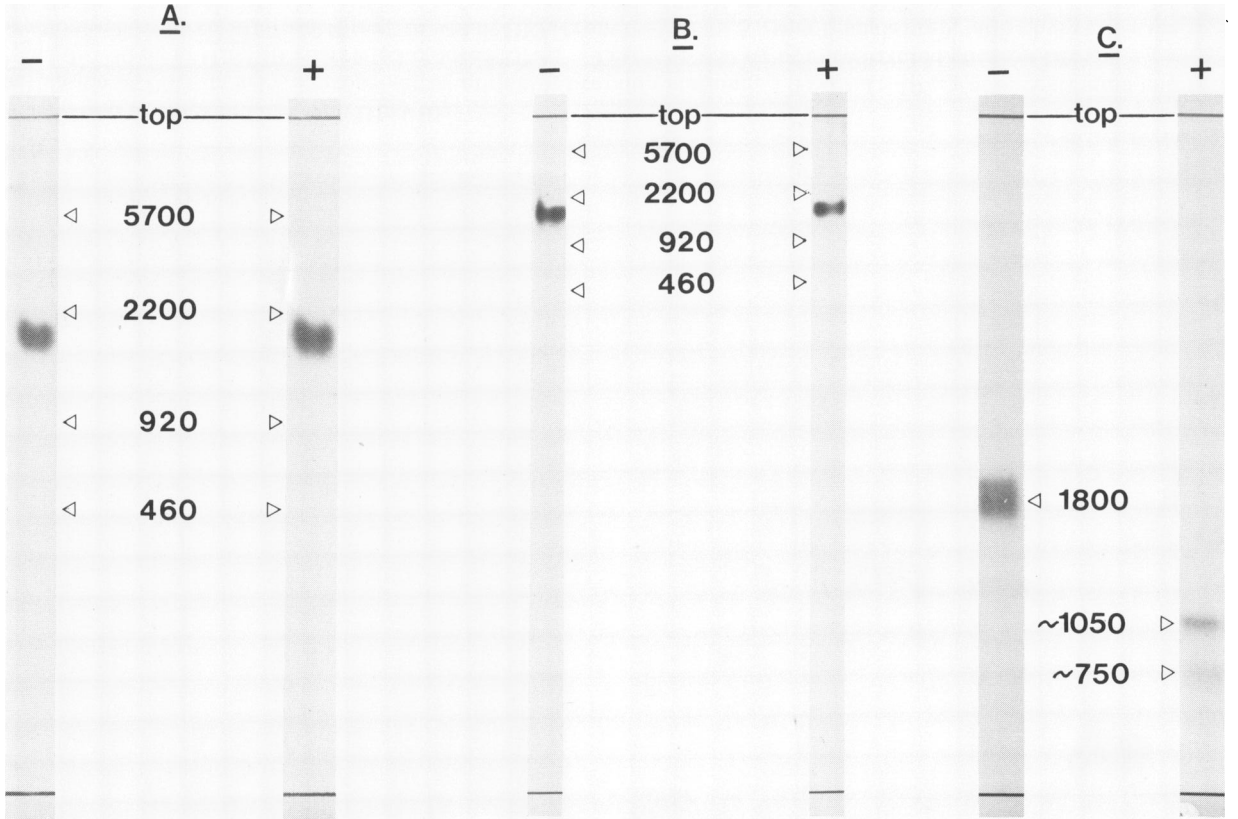

Fig. 1. Synthetic ovalbumin gene was incubated at 37°C either in the presence (+) or absence (-) of Hind III (A), Eco RI (B), and Hae III (C). Complete digestion was confirmed by inclusion of phage λ DNA (data not shown). Following electrophoresis through 1.4% agarose slab gels, the gels were dried and autoradiographed. The positions of Hind III fragments of phage PM2 DNA (represented as nucleotide base-pairs) are shown (A, B).

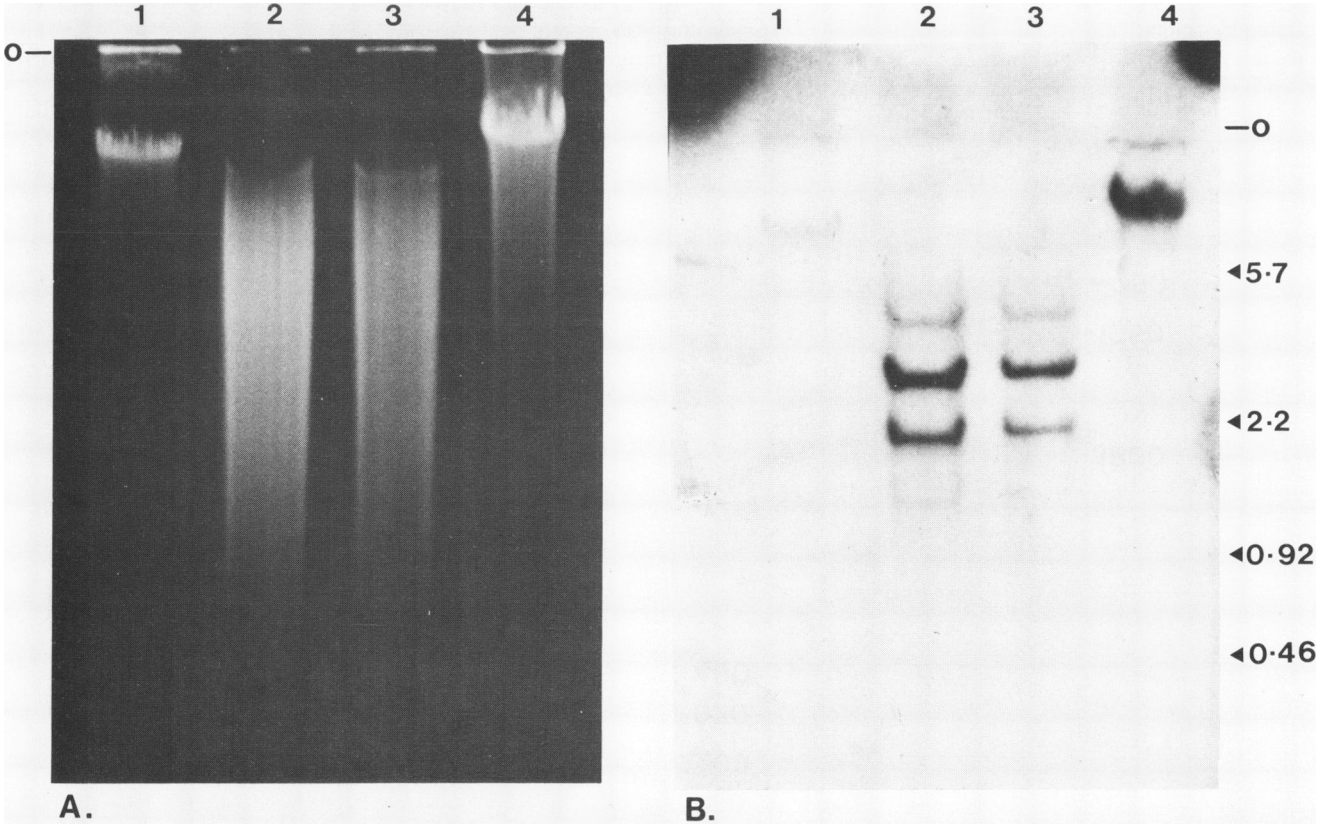

Fig. 2. Digestion of chicken DNA with Hind III.
A. Visualisation of DNA on a 1% agarose gel by ethidium bromide staining. Track 1: 10 μg of unrestricted chicken DNA. Tracks 2 and 3: 30 μg of Hind III restricted chicken DNA. Track 4: 30 μg of unrestricted chicken DNA.
B. Autoradiograph of nitrocellulose filter, to which DNA from the gel in A. was transferred, after hybridisation to ^{32}P-labelled ovalbumin mRNA fragments. The molecular sizes of Hind III digested PM2 DNA fragments are given in kilobase pairs.

chicken DNA produced the same pattern of major bands seen with the messenger derived probe (fig. 3).

The only difference observed was in the relative intensities of the three bands. This indicates that the observed bands are all ovalbumin specific and not due to a contaminant. The experiments described above to eliminate hybridisation or enzyme digestion artefacts were also done with this cloned probe. The unlikely possibility that some of the observed hybridisation was due to cross hybridisation to sequences in the parent pCR1 plasmid was eliminated by hybridising RNA transcribed from this plasmid to a filter carrying Hind III digested DNA. No hybridisation was detected.

Thus we conclude that the multiple bands seen on the autoradiographs are due to stringent hybridisation with part or all of the ovalbumin structural gene despite the fact that this sequence is not cut by Hind III. When the experiment was performed using Eco RI to restrict the chicken DNA the hybridisation of nitrocellulose filters to a plasmid derived probe again showed multiple bands (fig. 4). Three bands were seen whose sizes were estimated to be 6200, 1950 and 1400 base pairs.

There are several explanations of these results. Firstly, although the gene titration experiments estimated that there is only one ovalbumin gene per haploid genome (15, 16) it remains possible that the actual number is greater and that each of these genes gives rise to a different fragment on digestion. However, the smallest band in the Eco RI digest, and possibly others (fig. 2B), is smaller than the size of the transcribed gene as reflected in the size of ovalbumin mRNA (20, 21).

Secondly, it has been shown that the ribosomal RNA genes in Drosophila have inserts of non-coding DNA (22). This phenomenon could explain our observations since the inserted

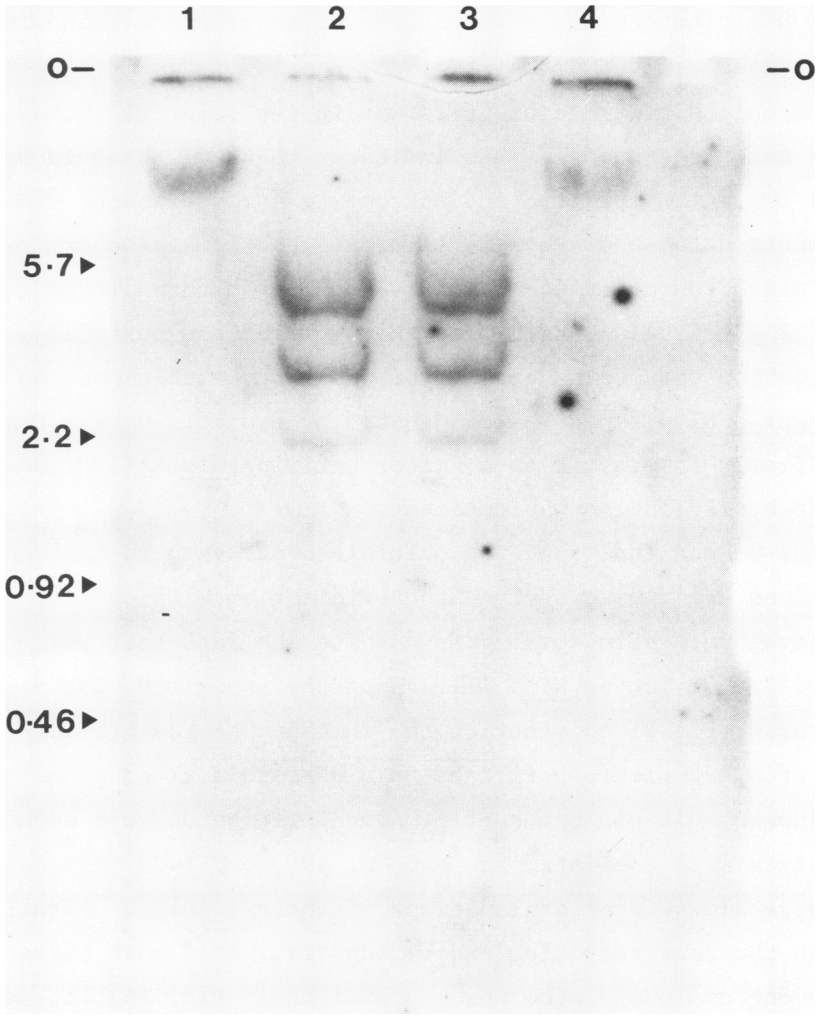

Fig. 3. Autoradiograph of a nitrocellulose filter bearing Hind III restricted chicken DNA after hybridisation to a ^{32}P-labelled RNA probe transcribed from the pCR1.0V2.1 plasmid, Track 1: 30 μg of undigested chicken DNA. Tracks 2 and 3: 30 μg of Hind III restricted chicken DNA, Track 4: 10 μg of undigested chicken DNA. The positions of Hind III digested PM2 DNA fragments are shown, together with their sizes in kilobase pairs.

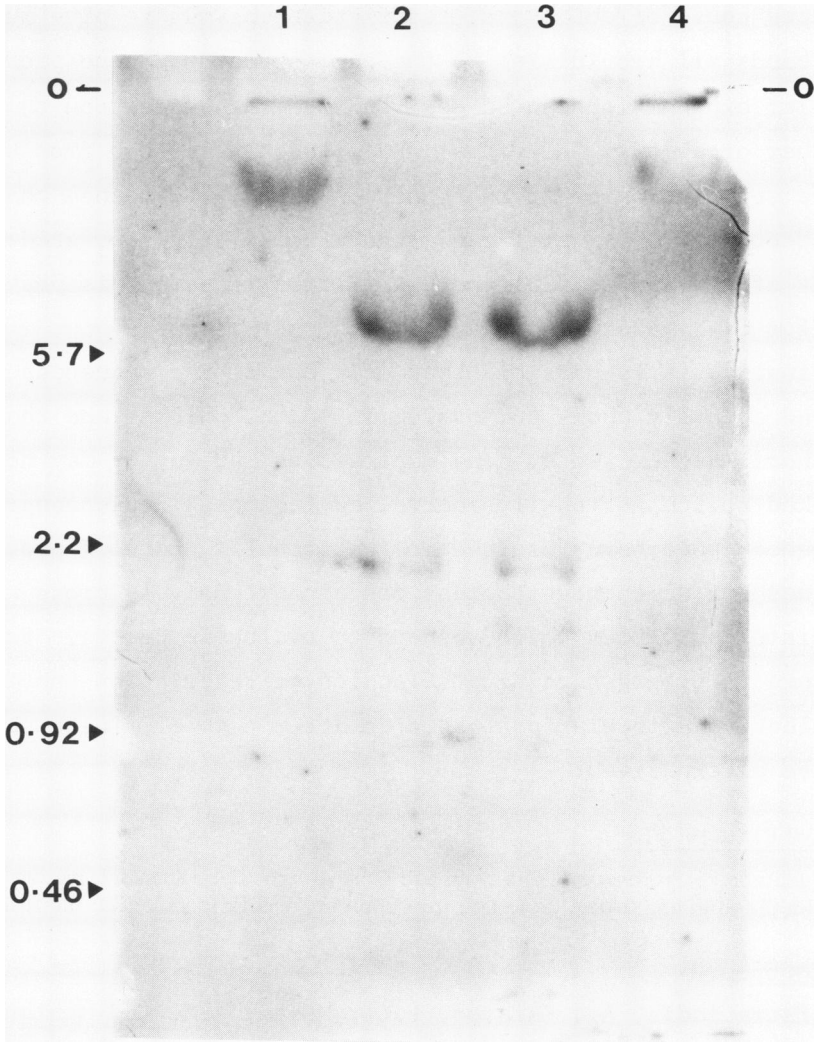

Fig. 4. Autoradiograph of a nitrocellulose filter bearing Eco RI restricted chicken DNA after hybridisation to a ^{32}P-labelled RNA probe transcribed from the pCR1.OV2.1 plasmid. Tracks 1 and 4: 30 μg of undigested chicken DNA, Tracks 2 and 3: 30 μg of Eco RI digested chicken DNA. The positions of Hind III digested PM2 DNA fragments are shown, together with their sizes in kilobase pairs.

sequences could be cut by the restriction enzymes to give multiple bands all hybridising to the same structural gene probe. A similar explanation can be derived from the suggestion that in higher organisms, individual mRNAs are transcribed from two or more separate parts of the genome. There is evidence that some adenovirus mRNAs are transcribed from widely separated regions of the genome (23) and a similar arrangement is postulated for SV40 mRNAs (24).

Finally, it is possible that only one of the sequences observed actually codes for ovalbumin in the chicken and that the others have arisen by gene duplication and may or may not be linked to the hormonally controlled regions. Evolutionary divergence could have resulted in functionally distinct proteins, the genes for which nevertheless contain extensively cross hybridising sequences which respond to ovalbumin probes. Experiments are in progress using a selection of restriction enzymes and selective molecular probes in an attempt to eliminate some of these alternatives.

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ABBREVIATIONS

Restriction Endonucleases:

Eco RI	Enzymes from plasmid-bearing E.coli.
Hind III	Hemophilus influenzae strain d enzyme.
Hae III	H. aegyptius enzyme.
Ovalbumin c.DNA	DNA complementary to ovalbumin mRNA.