The Ovalbumin Gene

INSERTION OF OVALBUMIN GENE SEQUENCES IN CHIMERIC BACTERIAL PLASMIDS*

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Double-stranded ovalbumin DNA was amplified and purified by the cloning of bacterial transformants. The double-stranded DNA was synthesized from a complete complementary DNA transcript of ovalbumin mRNA using Escherichia coli DNA polymerase I and the self-priming ability of the initial transcript. After S, nuclease treatment, poly(dA) was added to the 3′ termini with terminal deoxynucleotidyltransferase and the ovalbumin gene was hybridized to a linear plasmid DNA, pMB9, containing 3′-poly(dT) termini. This hybrid molecule was used to transform the E. coli strain X1849. The cloned transformants contained from 30 to 55% of the complete ovalbumin DNA as determined by hybridization with full length cDNAω. The length of the inserts was confirmed by treatment of the isolated plasmids with the restriction enzyme Hha I. Separation of the fragments by agarose gel electrophoresis showed that the amount of inserted DNA in the clones tested varied from 680 to 1090 base pairs.

EXPERIMENTAL PROCEDURES

Synthesis of Double-stranded Ovalbumin DNA—Ovalbumin mRNA was purified from hen egg yolk by methods previously described (22). The complementary DNA transcript was synthesized from ovalbumin mRNA using avian myeloblastosis virus RNA-directed DNA polymerase (reverse transcriptase) in a 1-ml reaction containing the following components: 50 mM Tris/HCl, pH 8.3, 20 mM β-mercaptoethanol, 10 mM MgCl2, 20 μg/ml of oligo(dT)20, 30 μg/ml of actinomycin D, 1 mM dCTP, 1 mM dGTP, 1 mM dITP, 1 mM dATP; 1 μCi [3H] dGTP (50 μCi/μmol), 98 μg/ml of mRNAω, 120 units/ml of reverse transcriptase. The mixture was incubated at 46°C for 15 min and the reaction terminated the addition of EDTA to a final concentration of 20 mM. Protein was removed by extraction with an equal volume of water-saturated phenol, and the soluble nucleotides were removed by chromatography on a Sephadex G-100 column (1 × 50 cm). The nucleic acid polymers were precipitated in 0.2 M ammonium acetate with 100 μg of Escherichia coli tRNA with 2 volumes of cold ethanol. The precipitate was resuspended in 0.5 ml of water and incubated for 20 min at 70°C in 0.3 N NaOH, 8 mM EDTA. The mixture

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† The abbreviations used are: EDTA, ethylenedinitrilo-tetraacetic acid tetrasodium salt; cDNAω, DNA complementary to ovalbumin mRNA; anti-cDNAω, DNA complementary to cDNAω, NTP, nucleotide pairs; pMB9, a chimeric pMB9 plasmid containing ovalbumin DNA; pDNA, plasmid DNA.
was then neutralized with 150 μl of 3 N HCl and again precipitated with ethanol after the addition of 200 μl of 1 M sodium acetate, pH 5.3. The precipitate was then redissolved in water at 0.1 μg/μl. However, cDNA to be used in hybridization studies was made as in Monahan et al. (23). This cDNA contains a hairpin loop at the 3' end and is a self-primering template for synthesis of a complementary strand by E. coli DNA polymerase I (6, 14, 15). Synthesis of the second strand of the ovalbumin structural gene (i.e., the coding strand of the structural gene) was carried out as previously described (6, 15). The DNA polymerase I, purified through the Sephadi G-100 step (24), was a gift from Dr. Roy Curtiss, University of Alabama School of Medicine (Birmingham) which had been supplemented with 100 μg/ml of dianminopimelic acid and 50 μg/ml of nalidixic acid. Twenty milliliters of cells were collected and resuspended in 3 ml of L-broth containing 100 μg/ml of dianminopimelic acid. The bacteria were incubated for 3 min, collected by centrifugation and resuspended in 3 ml of L-broth containing 100 μg/ml of dianminopimelic acid. The hairpin loop remained after the addition of 100 μg/ml of double-stranded DNA (6). The S1 nuclease, prepared by the method of Vogt (25), was a gift of C. Beard. After a 1-h incubation at 37°C the reaction was terminated by the addition of Tris base to a final concentration of 100 mM. Following extraction with phenol, the DNA was precipitated by the addition of 2 volumes of ethanol.

Poly(dA) Addition—The DNA pellet was resuspended at a concentration of 1 μg/ml in 50 mM KH2PO4, pH 8.8, 8 mM MgCl2, 1 mM mercaptoethanol, 50 μM dATP. (32) Poly(dA) (7 μCi/ml), with 2288 units/ml of terminal deoxynucleotidyl transferase (12.5 units/μg) in a 150-μl reaction. The enzyme was purified by a modification of the procedure of Chang and Bollum (26) in the laboratory of R. L. Rattiff with the addition of C. Marsono and C. Manske. The number of dA residues added to the 3' termini was calculated from the specific activity of the dATP. The addition of bases per 3' termini required approximately 30 min. The reaction was stopped by the addition of 20 mM EDTA and 200 mM sodium acetate, pH 5.4. Following the addition of RNA polymerase was added and the nucleic acid was precipitated with 2 volumes of ethanol.

Isolation and Cleavage of pMB9—The plasmid pMB9 was constructed in vitro from the tetracycline-resistance plasmid pSC101 and the colicin factor C81E1 in the laboratory of H. W. Boyer, University of California, San Francisco (27). The plasmid, which was grown in E. coli HBI129 and isolated as in Kutz et al. (28), and cleaved with Eco RI endonuclease and "tailed" with 100 poly(dT) residues as in Higuchi et al. (8), was a gift from R. Higuchi and M. Kamaromy.

Bacterial Transformation—Hybridization of the pMB9-(dT)100 (2 μg/ml) to the double-stranded ovalbumin DNA-(dT)100 (0.5 μg/ml) was performed in 10 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl. Following an incubation at 37°C for 30 min, the reaction was cooled gradually over 3 h to room temperature. The buffer was then adjusted to 50 mM CaCl2 and 8 mM MgCl2 prior to the transformation procedure.

E. coli strain X1849 (obtained as a gift from Dr. Roy Curtiss, University of Alabama School of Medicine in Birmingham) which had been supplemented with 100 μg/ml of dianminopimelic acid and 50 μg/ml of nalidixic acid. Twenty milliliters of cells were collected and resuspended in 20 ml of 50 mM CaCl2 for 15 min at 4°C. The cells were again collected, resuspended in 1 ml of cold 50 mM CaCl2 and added to 0.5 ml of the DNA solution at 4°C. After 15 min at 4°C the cells were heated to 37°C for 3 min, collected once again, and resuspended in 5 ml of L-broth containing 100 μg/ml of dianminopimelic acid. The bacteria were incubated for 30 min at 37°C, and spread on L agar Petri plates containing 15 μg/ml of tetracycline. All resistant colonies were transferred by a sterile tooth pick to a Millipore filter. The cells were then lysed with alkali and hybridized to 32P-labeled DNA transcribed from cDNA, as described by Grunstein and Hogness (31). The positive colonies from the filter hybridization were detected by autoradiography. The E. coli RNA polymerase used to synthesize the 32P-labeled DNA was a gift from R. Burgess, University of Wisconsin (32).

Restriction Enzyme Analysis—Positive bacterial colonies were grown in L-broth containing 50 μg/ml of dianminopimelic acid. Plasmid DNA was prepared by making a cleared lysate of the cells (28) followed by a phenol/chloroform (1:1) extraction as described above.

E. coli tRNA (40 μg/ml) was added as carrier, the solution made 0.3 M sodium acetate, pH 5.4, and the DNA precipitated with 2 volumes of ethanol. About 1 μg of DNA from each of the four clones was digested for 12 h at 37°C with 2 units of Hpa II restriction endonuclease (Bethesda Research Laboratories, Inc., Bethesda, Md.) in 15 mM Tris, pH 7.3, 5 mM MgCl2, and 5 mM 2-mercaptoethanol. SV40 DNA and plasmid pMB9 DNA were also digested as controls. The samples were electrophoresed on a 3% agarose slab gel (10 x 16 cm) in an apparatus made by Blain Craft Inc., Cold Spring Harbor, N. Y. The electrophoresis was performed in 50 mM Tris, pH 8.4, 20 mM sodium acetate, 18 mM NaCl, and 2 mM EDTA, and the gel was then stained with 5 μg/ml of ethidium bromide in electrophoresis buffer for 10 to 15 min. DNA was visualized with shortwave ultraviolet light.

Hybridization and Preparation of Probes—The [3H]cDNA (81), which is homologous to the coding strand for ovalbumin mRNA, was synthesized from purified hen ovalbumin mRNA by reverse transcription as previously described (23). The cDNA was approximately 1,800 nucleotides in length with a specific activity of 20 x 106 cpm/μg. This full length cDNA was separated by alkaline sucrose gradient centrifugation from incomplete transcripts.

To determine the length of the inserted ovalbumin DNA in the chimeric plasmids, the [3H]cDNA was hybridized to purified plasmid DNA. The purified DNA was sonicated to a size of 400 with a Sonifier cell disruptor made by Heat-Systems Ultrasonics, Inc. of Plainview, N.Y. The sonication of the covalently closed circular plasmid DNA was necessary to prevent snapshot during the hybridization experiments. The liquid hybridization conditions were similar to those earlier (34). There was 1.2 μg of the individually cloned chimeric plasmids in the 50-μl reaction mixture. This concentration is equivalent to approximately 200 Cpm/μl of [3H]cDNA. Each reaction had approximately 35,000 cpm of [3H]cDNA, which means that there was a 600- to 700-fold mass excess of the plasmid DNA in the reaction mixture. After incubation for 2 h at 68°C, the reaction was stopped by placing the vials in a mixture of dry ice/ethanol. The precipitate was then neutralized with 150 μl of 1 N HCl and again precipitated with 2 volumes of ethanol.

Results

The formation of the chimeric plasmid is essentially that described by Efstratiadis and co-workers (13) and Higuchi et al. (8), except that the partial digestion with A1 exonuclease was omitted in the poly(A) tailing of the DNA (35). Fig. 1 diagrammatically shows the procedures involved in the formation of the chimeric plasmid. The first step...
involves the synthesis of double-stranded cDNA, with Escherichia coli DNA polymerase I. No primer is necessary since the cDNA, is self-priming (10), as has been previously observed with globin cDNA (7–9). After cleavage with S1, poly(dA) was added to both ends of the cDNA, molecule with terminal deoxynucleotidyltransferase. After exposure of the plasmid pMB9 to Eco RI, the same enzyme was used to add poly(dT) to the 3’ end of the plasmid DNA molecule. The double-stranded cDNA, (dA), was then hybridized to pMB9-(dT), and used to transform the bacterial strain X1849. This technique has been used previously to obtain plasmids containing rabbit globin DNA (8, 13). The plasmid pMB9 has a dual advantage; it confers bacterial resistance to tetracycline, and has the ability to continue to replicate when bacterial growth is inhibited by chloramphenicol. When cell protein synthesis is inhibited the plasmid can comprise up to 40% of the total cellular DNA (36).

Fig. 2 shows the autoradiograph of the 26 clones that were tetracycline-resistant. The bacteria were grown on a lined Millipore filter, lysed with alkali, then hybridized with 32P-labeled cDNA, transcribed from cDNA,.. The labeled filter, which aids in numbering the clones, has been redrawn over the X-ray film. Thirteen clones hybridized to the probe. Four or five colonies which were strongly positive and were observed on a duplicate filter were studied in more detail. Plasmid DNA was purified for additional analysis from four clones which were designated, pOv1, pOv2, pOv3, and pOv4. The size of the inserted DNA was determined by agarose gel electrophoresis of the plasmids which had been digested with the restriction enzyme, Hha I (Fig. 3). SV40 DNA was digested with the restriction enzyme, Hpa I, into three fragments of 2075 NTP, 1925 NTP, and 1000 NTP (37). The size of the experimental bands was calculated from their mobilities relative to the three SV40 standards. The restriction enzyme, Hha I, has been shown by Maniatis et al. (13) to cut pMB9 into several fragments, with the largest fragment (950 NTP) containing the Eco RI site for foreign DNA insertion. The next four columns, pOv1, pOv2, pOv3, pOv4, are the digests of four separate plasmids that contain ovalbumin DNA. The far right column is a calibration standard of SV40 DNA digested with Hpa I. The three bands are 2075 NTP, 1925 NTP, and 1000 NTP in length. The photograph of the gel was cut into strips to allow room for labels in this figure.

![FIG. 2 (left). Autoradiograph of the transformed clones hybridized to 32P-labeled cDNA,.. The clones were grown on a Millipore filter containing a grid network. The lines have been redrawn over the autoradiograph.](image_url)

![FIG. 3 (right). Agarose slab gel of chimeric ovalbumin plasmids treated with the restriction enzyme Hha I. The DNA was stained with ethidium bromide and photographed with ultraviolet illumination. The left column is a control digestion of the plasmid without any inserted ovalbumin DNA. The arrow indicates the 950 NTP band that contains the Eco RI site for foreign DNA insertion. The next four columns, pOv1, pOv2, pOv3, pOv4, are the digests of four separate plasmids that contain ovalbumin DNA. The far right column is a calibration standard of SV40 DNA digested with Hpa I. The three bands are 2075 NTP, 1925 NTP, and 1000 NTP in length. The photograph of the gel was cut into strips to allow room for labels in this figure.](image_url)

### TABLE I

| Chimeric plasmid | Hha I fragment lengths (determined from gel) | Inserted DNA | Ovalbumin DNA (determined by hybridization) | Estimated poly(dA·dT) per terminus |
|-----------------|--------------------------------------------|--------------|-----------------------------------------|---------------------------------|
| pOv1            | 1675                                      | 735          | 555                                     | 95                             |
| pOv2            | 1630                                      | 680          | 550                                     | 65                             |
| pOv3            | 2040                                      | 1090         | 930                                     | 80                             |
| pOv4            | 1950                                      | 1000         | 790                                     | 105                            |

The transcription of the ovalbumin gene was studied in isolated bacterial minicells containing the chimeric plasmid. Hybridization to the total RNA isolated from the minicells showed that about 50% of the coding strand was transcribed while only 20% of the anticoding strand was transcribed for clone pOv4.2. The RNA complementary to the coding strand of the DNA (i.e. RNA with sequence homology to the mRNA) comprised 0.8% of the total RNA in the minicells. The in vitro transcription of this same plasmid DNA using purified E. coli RNA polymerase showed no preference for either the coding or anticoding strand of the inserted ovalbumin DNA.2

**DISCUSSION**

There are several different lines of evidence that show that

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ovalbumin DNA has been inserted and replicated in the plasmid pMB9. The first is the in situ hybridization of the individual colonies shown in Fig. 2. In addition, the purified plasmid DNA from transformants hybridized to cDNA, while the plasmid DNA did not. The correlation between the physical size of the inserted DNA as determined by gels and the length determined by hybridization to cDNA, argues that the larger size plasmids are produced by inserted ovalbumin DNA. The small differences between these two values probably reflects the oligo(dA:dT) regions that connect the plasmid and ovalbumin DNAs. Finally, using a [3H]cDNA, hybridization probe, we can detect the presence of mRNA sequences in RNA synthesized from plasmid DNA in vivo and in vitro.

None of the initial four clones characterized contain the entire sequence complementary to mRNA. The clones contain from 31 to 53% of the complete sequence of the ovalbumin structural gene. The partial inserts are easily explained by the omission of a sizing procedure during the preparation of the double-stranded cDNA. A complete copy of the double-stranded cDNA has been synthesized with viral RNA-dependent DNA polymerase (10). This DNA is presently being used to obtain a plasmid containing the entire mRNA sequence.

Bacterial cloning of eukaryotic DNA allows the study of both the expression and the structure of purified genes. Since the ovalbumin gene is hormonally responsive, amplification and purification of milligram amounts of the complete DNA sequence via the use of bacterial plasmids should facilitate the discussion of the steps involved in steroid hormone regulation of gene transcription. The role of DNA sequences in binding steroid receptor proteins, RNA polymerase, and chromosomal proteins can be evaluated. In addition, cleavage of the DNA by restriction enzymes has the potential of allowing the isolation and sequencing of specific regions of interest in the gene.

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