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Biochemistry

Chicken ovalbumin is synthesized and secreted by *Escherichia coli*

(recombinant DNA/gene expression/*lac* control/plasmids)

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ABSTRACT **By recombinant DNA methods, the chicken ovalbumin structural gene has been fused to *Escherichia coli lac* transcriptional and translational control regions. When a plasmid containing the hybrid gene was introduced into *E. coli*, a protein identified as ovalbumin by immunoreactivity and sodium dodecyl sulfate/polyacrylamide gel electrophoresis was synthesized. The chicken ovalbumin made in bacteria was full length (43,000 daltons) and constituted 1.5% of the cellular protein. In addition, the microbially synthesized ovalbumin was secreted through the cell membrane into the periplasmic space of *E. coli*. The ability of the *E. coli* secretory apparatus to recognize chicken ovalbumin, which is normally synthesized and secreted in hen oviducts, suggests that common features exist in the secretion-recognition mechanisms found in these two organisms. The bacterial synthesis of significant amounts of chicken ovalbumin demonstrates that the *E. coli* cellular machinery may be utilized to synthesize a higher eukaryotic protein which is relatively stable in the bacterial intracellular environment.**

Many of the potential benefits envisioned as a result of the application of recombinant DNA technology to the solution of medical problems require the insertion, into microorganisms, of genes coding for proteins normally found in higher eukaryotic organisms. Although it has been possible to clone a number of different higher eukaryotic genes containing the information necessary to code for proteins, reports of the expression of these proteins in bacteria have been limited to the human polypeptide hormone somatostatin (1) and rat proinsulin (2).

The somatostatin gene was chemically synthesized and the DNA sequence coding for its 14 amino acids was fused to the β-galactosidase structural gene on a plasmid. Yields of somatostatin varied from 0.001 to 0.03% of the total cellular protein. The rat proinsulin gene was inserted into a plasmid-borne penicillinase gene and the bacteria produced approximately 100 molecules of proinsulin per cell.

We report here the *in vitro* construction of a multicopy plasmid in which the *lac* operon control region was fused to a DNA sequence coding for chicken ovalbumin, a 43,000-dalton protein. This egg white protein is normally synthesized and secreted in the chicken oviduct. Bacteria containing the fused plasmid synthesized about 1.5% of their protein as full-length immunoreactive ovalbumin. The production of ovalbumin has been shown to be under *lac* control and the protein is stable *in vitro* to *Escherichia coli* proteases. Because *E. coli* is capable of secreting specific proteins, we also performed experiments to determine whether the *E. coli* secretory machinery can recognize the ovalbumin molecule and found that ovalbumin is secreted through the cell membrane into the periplasmic space.

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MATERIALS AND METHODS

Cells and DNA. *E. coli* HB101 (UC 6479) (3) was used in all experiments. The *lac* UV5 promotor plasmid pOP203 was constructed by F. Fuller, L. Johnsrud, and W. Gilbert (personal communication) by cloning a 203-base-pair *Hae* III fragment of *lac* DNA containing the promotor, ribosome binding site, and codons for the first seven amino acids of β-galactosidase into the tetracycline resistance plasmid pMB9 (4). The plasmid has a single *Eco*RI site located so that the *lac* promotor will direct transcription into any DNA fragment ligated into that site. The pOV230 plasmid was constructed by L. A. McReynolds, J. F. Catterall, and B. W. O'Malley (5) and was obtained in a cooperative laboratory arrangement. Plasmid DNA was prepared by the salt precipitation method of Guerry *et al.* (6) and further purified by cesium chloride/ethidium bromide density gradient centrifugation (7). Transformation was done essentially as described by Wensink *et al.* (8). Cell extracts were prepared by grinding with alumina, and protein concentrations were determined by the method of Lowry *et al.* (9) with bovine serum albumin as the standard.

Enzyme Reactions. All restriction endonucleases were purchased from New England BioLabs. *Eco*RI, Taq I, and *Hha* I digestions were done in reaction mixtures recommended by the supplier, except that autoclaved gelatin was substituted for bovine serum albumin. Alkaline phosphatase treatment of *Eco*RI-cut pOP203 DNA was carried out essentially as described by Ullrich *et al.* (10), except that the enzyme was preincubated at 70°C for 10 min prior to addition of DNA. T4 DNA polymerase-catalyzed fill-in of staggered ends was done in a reaction mixture described by Goulian *et al.* (11), containing 0.2 mM deoxynucleotide triphosphates.

*Eco*RI linkers, obtained from Collaborative Research, were phosphorylated by using polynucleotide kinase (12) and then heated to 70°C and slowly cooled to 4°C. The reaction mixture was then brought to 45 mM Tris·HCl (pH 7.8), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 15 mM dithiothreitol, 1 mM ATP, 20 μg of *Taq* I-cut pOV230 DNA per ml, and 14 units of T4 DNA ligase (New England BioLabs) per ml and incubated at 12.5°C overnight. Joining of the purified ovalbumin gene fragment to the alkaline phosphatase-treated pOP203 was also catalyzed by T4 DNA ligase (12).

Gel Electrophoresis. DNA was extracted from agarose gels by maceration and overnight incubation at 47°C in 10 mM Tris·HCl, pH 8.0/2 mM EDTA/1 M NaCl. The agarose was pelleted at 100,000 × *g* for 1 hr and the supernatant was phenol extracted, ether extracted, and ethanol precipitated.

Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis for protein separation was done as described by Laemmli (13) with a Bio-Rad slab gel apparatus. Samples were dissolved in an equal volume of sample buffer [61.5 mM Tris·HCl, pH 7.8/3% (wt/vol) $NaDodSO_4$/5% (vol/vol) 2-mer-

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

captoethanol/20% (vol/vol) glycerol] and heated at 100°C for 5 min (14).

Immunoassays. The *in situ* immunoassay was done as described by Skalka and Shapiro (15), with the N-Z bottom agar containing 25 μl of ovalbumin antisera (Antibodies, Inc., Davis, CA) per ml, 10 μg of tetracycline per ml, and 1 mM isopropylthiogalactoside.

Immunoreactive material in cell extracts was precipitated for gel analysis by adding antiserum to extracts. The mixtures were allowed to stand for 60 min at room temperature and then overnight at 4°C; the precipitates were washed twice with 25 mM Tris·HCl, pH 7.4/137 mM NaCl/5 mM KCl.

Containment. All recombinant DNA experiments were done under P2-EK1 containment.

RESULTS

Construction of the *lac*–ovalbumin fused plasmid, pUC1001, proceeded as shown in Fig. 1. In our construction we cut the pOP203 plasmid with *Eco*RI and treated the resulting linear molecule with alkaline phosphatase, removing the 5′-phosphate groups to prevent recircularization of the pOP203 vector DNA during ligation (10).

The pOV230 plasmid was constructed with a pMB9 vector and contained nearly all of the ovalbumin mRNA sequence, including all of the information required to code for the amino acid sequence of chicken ovalbumin. The sequence of the ovalbumin gene insert in pOV230 (16) revealed a unique *Taq* I restriction endonuclease site 25 base pairs to the 5′ side of the ovalbumin initiator AUG. Fusion of bacterial control regions at this site would allow expression of the entire ovalbumin structural gene sequence. As shown in Fig. 1, an additional *Taq* I site is located approximately 250 base pairs outside of the ovalbumin insert such that *Taq* I digestion yields a DNA fragment of about 2200 base pairs containing the entire ovalbumin structural gene.

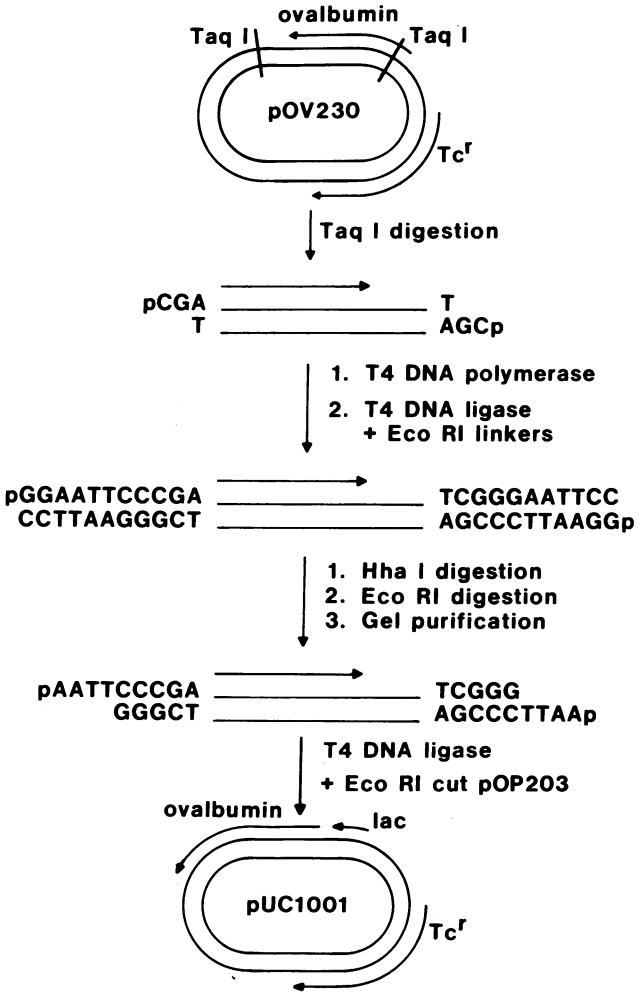

FIG. 1. Outline of steps used to fuse *lac* control elements to the ovalbumin gene, resulting in plasmid pUC1001.

Because the sequences of the *lac* control region inserted into pOP203 (17, 18) and of the ovalbumin gene insert in pOV230 are known, our strategy was to fuse the *lac* and ovalbumin DNAs in a manner that would maintain the translational reading frame, assuming protein synthesis began at the β-galactosidase initiation codon. There are several ways in which this can be done, but we chose to fill in the stagger-ended *Taq* I fragments with DNA polymerase and then ligate synthetic *Eco*RI octamer linkers to the blunt ends as shown in Fig. 1. A large excess of linker molecules was used in the reaction to prevent ligation of the filled-in *Taq* I fragments to each other. The linked fragments were digested with *Eco*RI to generate 5′ staggered ends. Gel purification was facilitated by digestion with *Hha* I to cut a pOV230 fragment that otherwise migrated with the ovalbumin gene.

After elution from the gel, the purified pOV230 DNA fragment was ligated to the alkaline phosphatase-treated pOP203 plasmid vector. This ligated DNA was then used to transform *E. coli* HB101, and tetracycline-resistant colonies were selected. The transformants were assayed for ovalbumin production by an *in situ* immunoassay. Colonies were lysed with lysozyme and Sarkosyl after growth on agar containing ovalbumin antiserum, isopropylthiogalactoside, and tetracycline. Easily discernible precipitin rings formed around several of the colonies, indicating that they were producing substantial amounts of ovalbumin.

In order to verify the key features of the plasmid DNA structure in the ovalbumin-producing bacteria, plasmid DNA from eight transformants was subjected to restriction digestion with *Hae* III alone and with both *Hae* III and *Eco*RI and analyzed by polyacrylamide gel electrophoresis. Fig. 2 illustrates the expected *Hae* III and *Eco*RI cleavage sites in our constructed plasmids. Construction *A* would be expected to synthesize ovalbumin whereas construction *B* would not be expected to synthesize it. *Hae* III digestion would allow determination of the orientation in which the ovalbumin gene is inserted relative to the *lac* control region; *Hae* III/*Eco*RI double digestion would show whether both *Eco*RI sites are present.

Fig. 3 shows the results of these restriction digestion analyses for eight transformants. Lanes 17 and 18 contained the *Hae* III digest and the *Hae* III/*Eco*RI double digest of pOP203. The largest fragment in lane 17 contained the *lac* control region and was 1052 base pairs long. This fragment was cut by *Eco*RI to yield the two fragments, 350 and 700 base pairs, seen in lane 18.

Lanes 1, 3, 5, 7, and 9 contained plasmid DNAs in orientation *A* (Fig. 2); lane 11 contained plasmid DNA in orientation *B*. As shown in lanes 2, 4, 6, 8, 10, and 12, these transformants all contained two *Eco*RI sites.

Lane 13 contained a plasmid in which a deletion apparently occurred and could be explained if the larger band in a plasmid with the ovalbumin gene in orientation *A* had lost some DNA, giving rise to the observed doublet at about 1600 base pairs. The double digest in lane 14 indicates that one of the *Eco*RI sites had been lost, suggesting that a deletion in the 1800-base-pair fragment included the *Eco*RI site. Lanes 15 and 16 contained a plasmid in which a deletion occurred in the pOP203 vector.

The *in situ* immunoassay results showed that colonies con-

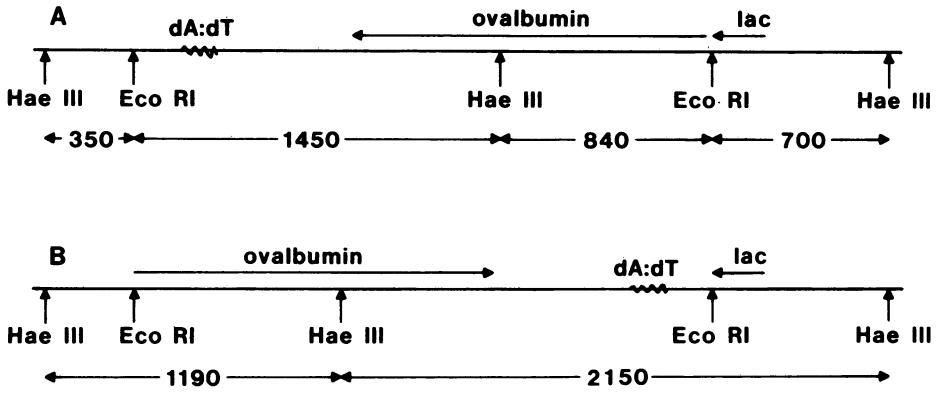

FIG. 2. Restriction digestion maps of plasmids containing the ovalbumin gene, showing the two possible orientations of that gene relative to the *lac* control elements.

taining the plasmids whose *Hae* III digestion patterns are shown in lanes 1, 5, 7, 9, and 13 of Fig. 3 produced ovalbumin. All of these plasmids had the ovalbumin gene inserted in orientation *A*. The plasmid in lane 3 also was in orientation *A* but did not produce ovalbumin, perhaps due to a frame shift caused by the addition or deletion of bases during the biochemical manipulations required for fusion.

Fig. 4 is a photograph of a Coomassie blue-stained NaDodSO$_4$/polyacrylamide gel in which several standards as well as immunoprecipitated ovalbumin had been electrophoresed. Lane 2 contained an anti-ovalbumin immunoprecipitate of an *E. coli* HB101 (pUC1001) extract. The top band is immunoglobulin heavy chain (50,000 daltons); the bottom band is immunoglobulin light chain (23,500 daltons). The dark band between the heavy and light chains in lane 2 is the *E. coli* ovalbumin. Although several other very faint bands are visible, it is clear that the immunoprecipitation is specific.

Lane 1 in Fig. 4 contained an immunoprecipitated *E. coli* HB101 (pUC1001) extract that had been subjected to incubation for 4 hr at 37°C. The size of the *E. coli* ovalbumin was not reduced by this self-digestion. Lanes 3, 4, and 5 contained protein standards to assist in the determination of the molecular weight of *E. coli* ovalbumin. Lane 3 contained alkaline phosphatase (43,000 daltons), lane 4 contained purified egg white ovalbumin, and lane 5 contained periodate-treated (19) egg white ovalbumin. Whereas the untreated egg ovalbumin migrated slightly faster than the *E. coli* ovalbumin, gentle treatment with periodate, which should remove carbohydrate residues, caused them to migrate at the same rate.

By using a gel diffusion immunoassay with egg white ovalbumin as a standard it was found that between 1.35 and 1.6% of the cellular protein was ovalbumin. Spectrophotometric scanning of Coomassie blue-stained NaDodSO$_4$/polyacrylamide gels also indicated that about 1% of the cellular protein was ovalbumin.

We found about a 50% decrease in the amount of ovalbumin produced in the absence of isopropylthiogalactoside, indicating that synthesis is under *lac* control. We expected only a small decrease because the multicopy pUC1001 plasmids contain functional *lac* operator, resulting in nearly constitutive levels of transcription in the absence of inducer.

To determine whether *E. coli* secreted ovalbumin, bacteria containing the pUC1001 plasmid were grown in the presence of isopropylthiogalactoside and tetracycline. The cells were treated with lysozyme and EDTA to give protoplasts. Centrifugation of the protoplast preparation yielded a supernatant fraction containing periplasmic proteins and a pellet containing protoplasts. β-Galactosidase was used as a marker for nonsecreted proteins and alkaline phosphatase was used as a marker for periplasmic proteins.

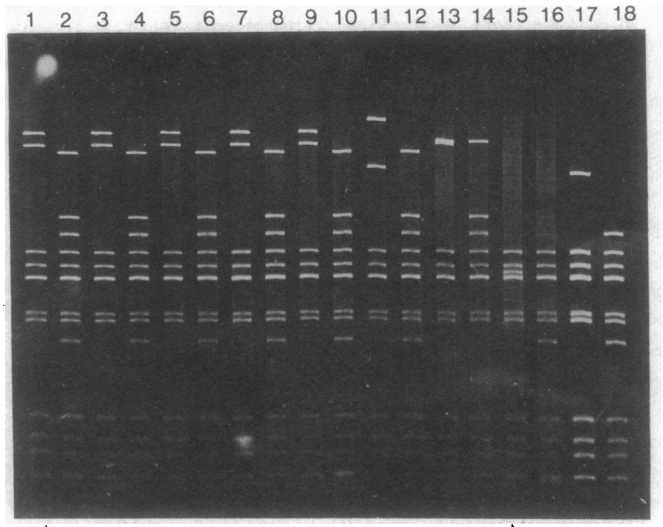

FIG. 3. Polyacrylamide gel electrophoresis of plasmid DNA restriction digestion fragments. The odd-numbered lanes are *Hae* III plasmid digestions and the even-numbered (n + 1) lanes are the same plasmid subjected to *Hae* III/*Eco*RI double digestion.

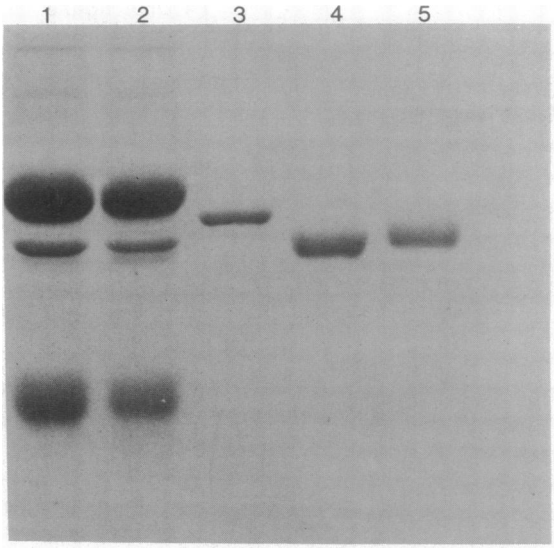

FIG. 4. Coomassie blue-stained NaDodSO$_4$/polyacrylamide gel. Lanes: 1 and 2, *E. coli* extracts precipitated with ovalbumin antiserum; 3, alkaline phosphatase; 4, purified egg white ovalbumin; 5, periodate-treated egg white ovalbumin.

Table 1. Results of protoplasting experiment

Fraction	β-Galactosidase activity		Alkaline phosphatase activity		Ovalbumin immunoreactivity	
	Units	% total	Units	% total	μg	% total
Periplasmic	1,005	3.7	32.24	73.4	620	48.6
Protoplast	26,189	96.3	11.7	26.6	656	51.4

Protoplasts of HB101 (pUC1001) were prepared essentially as described by Malamy and Horecker (20). β-Galactosidase and alkaline phosphatase were assayed as described (21, 22). Units are defined as the enzyme activities required to produce an absorbance change of 1.0/hr; the units shown represent the total activities from a 600-ml broth culture.

Table 1 shows the results of a protoplasting experiment. More than 96% of the β-galactosidase activity was in the protoplast fraction, indicating less than 4% leakage of this nonsecreted protein into the periplasmic fraction. Less than 75% of the alkaline phosphatase activity was in the periplasmic fraction, suggesting that some of the periplasmic protein is not released into the supernatant. Table 1 shows that approximately 50% of the ovalbumin was found in the periplasmic fraction and 50% in the protoplast fraction. In view of the extremely low level of β-galactosidase leakage into the periplasmic fraction, this indicates that the chicken ovalbumin was actively secreted by *E. coli*, although perhaps not as efficiently as *E. coli* secretory proteins such as alkaline phosphatase. In no case was any immunoreactive ovalbumin found in the growth medium. Na-DodSO$_4$/polyacrylamide gel electrophoresis of anti-ovalbumin immunoprecipitated periplasmic and protoplast fractions has shown that the secreted and nonsecreted ovalbumin have the same molecular weight.

DISCUSSION

We have shown that full-length ovalbumin molecules are synthesized in bacteria from information encoded in the pUC1001 plasmid. Because the *E. coli* ovalbumin expression from pUC1001 is induced by isopropylthiogalactoside, we believe that ovalbumin mRNA synthesis is initiated at the plasmid *lac* promotor. From Fig. 5, showing the first 107 bases of this mRNA, it can be seen that if protein synthesis is initiated at the β-galactosidase AUG (position 39) the ovalbumin molecule will be synthesized with 18 extra amino acids on its amino terminus. Addition or deletion of bases during the *in vitro* construction of pUC1001 would result in a frame shift, and ovalbumin would not be expressed. This seems to be the most likely explanation for lack of expression from the plasmid in lane 3 of Fig. 3 and, if true, would demonstrate that protein synthesis is initiated at the β-galactosidase AUG.

Our data, however, do not allow us to conclude directly whether the β-galactosidase protein synthesis initiation site is used for ovalbumin expression. The results from polyacrylamide gel electrophoresis show that the *E. coli* ovalbumin has a molecular weight of about 43,000, but this value is dependent upon standards whose gel migration characteristics have not been extensively characterized. It is possible that the 18-amino acid tail is synthesized and then cleaved from the molecule by intracellular proteases. This question will have to be looked at in more detail, however, before any firm conclusions may be drawn.

Although 1.5% of the cellular protein is ovalbumin in *E. coli* carrying the pUC1001 plasmid, this level is considerably lower than the level theoretically attainable in this system. We have determined (unpublished data) that there are between 25 and 30 copies of the pUC1001 plasmid per chromosome equivalent in *E. coli*. It is known that β-galactosidase, with one gene copy per chromosome equivalent, is expressed as 2% of the total *E. coli* protein in fully induced cultures (23). Therefore, considering the gene copy numbers as well as the differences in molecular weight between ovalbumin and β-galactosidase, we would expect approximately 19% of the total protein in our pUC1001-containing bacteria to be ovalbumin. In fact, although a significant amount of ovalbumin was synthesized, it was less than 10% of the theoretically predicted amount.

The most probable explanations for lower-than-theoretical levels of ovalbumin in cells carrying the pUC1001 plasmid are inefficient transcription, inefficient translation, and proteolytic degradation of ovalbumin molecules.

It has been reported that, when a *lac* promotor with the UV5 (CAP-independent) mutation is cloned onto a multicopy plasmid, it functions with full efficiency (24). Thus, it is also likely that transcription is fully efficient in the case of pUC1001. Inefficient translation of the ovalbumin mRNA may result from either inefficient initiation or inefficient elongation. It is known that at least 7 times the normal level of β-galactosidase can be made when the gene is on a multicopy plasmid (25). Therefore, it is likely that protein synthesis initiation is not limiting in our system. The rate of elongation of mRNA translation will be dependent upon many factors, one of which is the availability of aminoacyl-tRNAs with anticodons complementary to the codons on the mRNA. The sequence data from several prokaryotic and eukaryotic genes, including ovalbumin, have revealed nonrandom utilization of synonymous codons (16, 26, 27). Although not enough data have been collected to permit

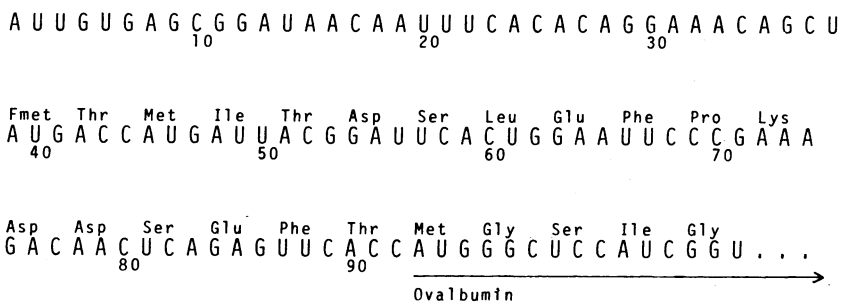

FIG. 5. Predicted pUC1001 mRNA sequence initiated at the *lac* promotor and predicted amino acid sequence initiated at the β-galactosidase AUG.

any generalizations, it is probable that some of the tRNA isoaccepting species required in relatively large amounts for the translation of ovalbumin are present in *E. coli* in low concentrations, thus limiting synthesis.

The third explanation for lower-than-theoretical ovalbumin production is proteolytic degradation. This may occur either with nascent polypeptide chains on polysomes or with mature ovalbumin molecules, or both. Our self-digestion data with *E. coli* cell extracts suggest that the mature ovalbumin molecules are stable to proteolytic digestion *in vitro*, but our data do not extend to the stability of nascent polypeptide chains.

The finding that ovalbumin molecules synthesized in *E. coli* are secreted into the periplasmic space suggests that common features exist in the secretion-recognition mechanisms found in chicken oviducts and in *E. coli*. More experiments will have to be done in order to determine what structural features of the bacterial ovalbumin cause it to be secreted. The ovalbumin naturally secreted from chicken oviducts is a complex protein, containing an amino-terminal acetyl group, carbohydrate residues, and phosphate residues. It is not clear what role, if any, these post-translational modifications play in secretion, and it has not been determined whether or not the bacterial ovalbumin is modified.

Whatever the mechanism for ovalbumin secretion in the hen oviduct, it would now appear that some aspects are common among living organisms. In this regard it should be noted that the secretory "signal" for chicken ovalbumin is probably not a hydrophobic amino-terminal sequence (28), as is the case with many secretory proteins (29, 30, 31). Thus, there may be additional structural features that are generally recognized for secretion.

Although further work is needed to maximize the synthesis of eukaryotic proteins in bacteria, the microbial production of significant amounts of chicken ovalbumin reported here demonstrates that the *E. coli* cellular machinery may be utilized to synthesize a eukaryotic protein that is stable in the bacterial intracellular environment. There is no reason to believe that the synthesis of other high molecular weight animal proteins will be any more difficult than that of ovalbumin.

Note Added in Proof. Results similar to those presented here involving synthesis of an ovalbumin-like protein by *E. coli* harboring a recombinant plasmid have recently been reported (32).

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