
Definition of the ovalbumin gene promoter by transfer of an ovalglobin fusion gene into cultured cells

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ABSTRACT

In order to study the initiation of transcription from the ovalbumin gene promoter, we constructed a hybrid gene (ovalglobin) in which 753 bps of ovalbumin gene 5'-flanking sequence were joined to the chicken adult β -globin gene. When transfected into HeLa S3 cells, ovalglobin gene transcription initiated at the ovalbumin gene cap site, as measured by S1 nuclease and primer extension analysis. Deletion of 5'-flanking sequences to position -95 had little effect on transcription; deletion to -77 reduced transcription to about 20% of the wild type level and deletion to -48 reduced the level to about 2%. A deletion to -24, removing the sequence TATATAT, abolished transcription entirely. Hormonal regulation of the ovalglobin gene was observed when primary oviduct cells were used as recipients for DNA transfection. Under these conditions, addition of progesterone increased the level of ovalglobin transcripts to more than 10 times the uninduced level.

INTRODUCTION

Biologists have long been interested in the mechanisms of gene regulation. Genes of a given organism may be turned on or off in response to an extracellular signal, or may be modulated as part of a developmental program. In recent years a variety of regulated genes have been cloned and analyzed, but distinct regulatory mechanisms have been uncovered in very few cases.

The functions of several cloned genes which are transcribed by RNA polymerase II have been studied by transcription in vitro of linear DNA templates using crude extracts of HeLa cells (1,2). This in vitro transcription is nonspecific, in that it takes place with genes which are normally expressed only in specific differentiated tissues. More recently, the functions of cloned genes have been studied by transferring them into cells in culture (3-7). The cloned genes are thus expressed in an environment which more closely resembles their in vivo state.

In both the in vitro and in vivo studies, the DNA sequences necessary for the initiation of transcription were defined by examining the effects of

specific mutations created in vitro. The transcription of linear templates in vitro requires only the TATA box, a consensus sequence located ~30 base pairs 5' to the mRNA cap site (8-12). The sequence requirements for transcription in gene transfer experiments are more extensive, and generally involve DNA sequences 100 bps or more 5' of the cap site (3,13-20).

These studies have analyzed the unregulated expression of genes transferred into undifferentiated, usually heterologous continuous cell lines. The regulated expression of transferred genes has been achieved in several systems such as the glucocorticoid regulation of the rat α_{2u} globulin gene (21), cadmium regulation of the mouse metallothionein gene (22), the induction of mouse β -globin gene transcription in differentiating Friend cells (23), and estrogen regulation of the chicken lysozyme gene (24). In several of these studies, DNA sequences upstream of the cap site were implicated in the regulation of transcription (23,24).

The particular model system for the regulation of gene expression that we have been studying is the induction of specific protein synthesis in the chicken oviduct by steroid hormones, which cause a large increase in the synthesis of many readily identifiable proteins, especially the major egg white proteins (25,26). For several of these proteins, the induction is due to an increase in the synthesis of their respective mRNA molecules (27-32). We have been particularly interested in the regulation of the ovalbumin gene, whose transcript is undetectable in unstimulated oviducts, but which makes up 50% of the mRNA population in oviducts stimulated by progesterone (30). The increase in ovalbumin gene transcripts is mostly due to an increase in the rate of transcription of the ovalbumin gene (33). Recent evidence suggests that DNA sequences located just 5' of the ovalbumin cap site are important for this increase in transcription. These sequences are thought to be sites at which specific hormone receptor molecules act to modulate transcription (29,34,35).

We describe here experiments designed to identify promoter elements within 5'-flanking region of the ovalbumin gene. To do this, we constructed a fusion of the ovalbumin gene 5'-flanking region and the chicken adult β -globin gene, and cloned this hybrid (ovalglobin) into a pBR322-SV40 vector. The transcription of the ovalglobin gene was characterized by transfer into HeLa S3 cells, in which the ovalglobin gene is expressed in a nonregulated fashion. By this method, we identified DNA sequences 5' of the cap site necessary for the constitutive function of the ovalbumin gene promoter. Finally, as a first step toward analyzing DNA sequences necessary for steroid regulation of ovalbumin gene transcription, we showed that the ovalglobin recombinant is transcribed

and regulated by progesterone following transient transformation of primary chicken oviduct cells.

MATERIALS AND METHODS

Enzymes

The following were purchased from Bethesda Research Laboratories: T4 polynucleotide kinase; bacterial alkaline phosphatase; T4 DNA ligase; Bal 31 exonuclease; and the restriction endonucleases Alu I, Ava I, Bgl II, Cla I, Eco RI, Hae III, Hpa II, Pvu II, and Taq I. E. coli DNA ligase and restriction endonuclease Sau 96-1 were purchased from New England Biolabs. E. coli DNA polymerase (Klenow) was purchased from Boehringer-Mannheim. S1 nuclease was purchased from P.L. Biochemicals, and AMV reverse transcriptase from Life Sciences Inc.

Cell Culture and DNA Transfection

HeLa S3 cells were obtained from the American Type Culture Collection, and were cultured as monolayers in Dulbecco's minimal essential medium with high glucose (DMEM-HG) containing 50 U/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf serum. Primary oviduct cultures were prepared as described (36), and were maintained in DMEM-HG containing 50 U/ml penicillin, 50 µg/ml streptomycin, 5 µg/ml of insulin and 5% horse serum. T47D cells were obtained from K. Horwitz, and maintained as described (37).

Cell lines were transfected one day after a 1:4 passage. Calcium phosphate-DNA coprecipitates (38) were prepared by mixing 20 µg of cloned DNA with 0.5 ml of a 2X HEPES buffer (containing, per liter: 10 g HEPES, 16 g NaCl, 0.74 g KCl, 0.22 g Na₂HPO₄, and 2 g dextrose; pH to 7.05). While bubbling air through the DNA/HEPES mixture, 0.5 ml of 0.25 M CaCl₂ was then added dropwise. The cocktail was thoroughly vortexed and then allowed to stand at room temperature for 30 min. The coprecipitate was added directly to the culture medium (1 ml/10 ml of culture medium) and left for 4-7 hr at 37°C. The medium was then removed and replaced with 25% DMSO in DMEM-HG (without serum) for 4 min at room temperature (39). The DMSO solution was removed, the monolayer was washed once with medium (containing serum) and then fresh medium was added to the cells for a further 40 hr incubation at 37°C. Primary oviduct cells were transfected 4-5 days after plating in the same way, except that the DMSO shock was 5% at 37°C for 15 min. Fresh medium with or without 10⁻⁷ M progesterone was then added for an additional 48 hr incubation at 37°C.

RNA Harvest

The procedure is essentially that of Sherrer (40). Medium was removed

from the monolayer and replaced with 10 ml of ice-cold Hank's incomplete balanced salt solution. The cells were scraped from the monolayer with a rubber policeman, poured into a 50 ml polypropylene centrifuge tube and collected by centrifugation. The cell pellet was suspended in 10 ml of 50 mM Na acetate, pH 5, and then lysed by the addition of SDS to 1%. Immediately, hot (60°C) phenol saturated in 50 mM Na acetate, pH 5, was added, and the aqueous phase was then extracted at 60°C for 5 min with frequent vortexing. The tubes were chilled at 0°C for 20 min and centrifuged for 20 min at 4,000 x g. The aqueous phase plus the interface was then subjected to a second hot phenol extraction exactly as described above. The second aqueous phase was made 0.25 M in NaCl, and the RNA was collected by ethanol precipitation. Where indicated, poly(A)⁺ RNA was isolated from the total RNA by affinity chromatography on oligo(dT) cellulose, as described by Aviv and Leder (41).

Construction of the Ovalglobin-SV40 Recombinant

Two DNA fragments were purified: a 794 bp Hae III-Taq I fragment of the ovalbumin gene containing 753 bp of 5'-flanking sequence and 41 bp of the first exon, and the 1364 bp Hpa II fragment of the chicken adult β -globin gene extending from position +115 (within the first exon) to position +1479 (within the third exon) (42). These fragments were joined with E. coli ligase, and then made blunt-ended by treatment with E. coli DNA polymerase I (Klenow) in the presence of dGTP and dCTP. The ligated and blunt-ended fragments were ligated to [³²P]-labelled Eco RI linkers [d(GGAATTCC); obtained from Collaborative Research], digested with Eco RI, and electrophoresed on a 4% polyacrylamide preparative gel. A fragment of the expected size (2166 bp) was detected by autoradiography, eluted from the gel slice, and ligated to Eco RI digested pBR322. After transformation of E. coli RRI (43), ampicillin-resistant colonies were picked onto replicate nitrocellulose filters and hybridized to both the 794 bp ovalbumin and 1364 bp β -globin fragments labelled with [³²P] by nick translation (44,45). Colonies that hybridized with both probes were further characterized by isolation of their plasmid DNAs and restriction endonuclease analysis to verify the presence of an ovalglobin insert with the expected structure. The ovalglobin insert was then purified by Eco RI digest followed by preparative acrylamide gel electrophoresis, and cloned into Eco RI linearized pSV-RI. Clones with the plasmid pSV.OG of the desired orientation (Fig. 1b) were identified by Pst I digests of small cleared lysates prepared as described by Birnboim and Doly (46).

Preparation of 5' End-Labelled DNA Probes

The DNA probe for mapping the 5' end of the ovalglobin transcript was pre-

pared by digesting the 873 bp Pvu II-Eco RI fragment of the ovalglobin gene with Sau 96-1, then 5' end-labelling the mixture of fragments with [³²P] using T₄ polynucleotide kinase as described (47). The 215 bp fragment spanning the 5' end of the ovalglobin gene was then isolated by preparative polyacrylamide gel electrophoresis. Where indicated, this probe was strand separated according to the method of Grosveld *et al.* (48). To map intron A, a 1537 bp Ava I to Bam HI fragment of the ovalglobin gene (1162 bp from the 5' half of the ovalglobin gene plus 375 bp of pBR322) was 5' end-labelled with [³²P] and then digested with Eco RI. The 1162 bp Ava I-Eco RI fragment was then isolated by preparative polyacrylamide gel electrophoresis (Fig. 2a). To map intron B, the 1364 bp Hpa II fragment of the chicken β-globin gene was 5' end-labelled with [³²P], digested with Bgl II, and fractionated on a polyacrylamide preparative gel to isolate the 899 bp Bgl II-Hpa II fragment (Fig. 2a).

S1-Nuclease Mapping

Ten to 50 ng (10⁵-10⁶ dpm) of 5' end-labelled fragment were mixed with RNA in 100 μl hybridizations containing 70% formamide (Fluka), 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA. After heating to 80°C for 10 min, hybrids were allowed to form for 12-16 hrs at 52°C (for the 215 bp Sau 96-1 probe) or at 57°C (for the 1162 bp Eco RI-Ava I and 899 bp Bgl II-Hpa II probes). The hybridizations were then quick-frozen at -70°C, thawed on ice, and brought to 500 μl by the addition of ice-cold H₂O and concentrated S1-nuclease buffer. The S1 reaction contained 0.2 M Na Acetate, pH 4.5, 0.4 M NaCl, 2 mM ZnCl₂ and 500 units of S1-nuclease. After incubation for 90 min at 37°C the S1-resistant nucleic acids were collected by precipitation with ethanol and centrifugation. The pellets were dissolved in 10 μl of 0.1 M NaOH and heated to 68°C for 5 min. Ten μl of dye mix (10 M Urea, 0.05% brom phenol blue, 0.05% xylene cyanol) were added and heating at 68°C was continued for 5 min. The samples were then electrophoresed on a 7 M urea-polyacrylamide gel at 200 v for 2-3 hrs. Labelled DNA fragments were detected by exposure of the gel to Kodak XAR-5 film at -20°C.

Primer Extension

This was done essentially as described by McKnight *et al.* (19). The primer was a 40-bp fragment derived by Alu I digest of the 215 bp Sau 96-1 fragment of the ovalglobin gene and 5' end-labelled with [³²P] at the Sau 96-1 site (Fig. 3a). Two ng of primer (~10⁵ dpm) were mixed with RNA in a 10 μl hybridization containing 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA. The hybridization was sealed in a capillary, heated to 100°C for 10 min and then incubated at 68°C for 12 hrs. Extension was done in a 50 μl reaction contain-

ing 10 mM Tris-HCl, pH 8.3, 10 mM DTT, 8 mM MgCl₂, 50 µg/ml actinomycin D, 1 mM each of dATP, dGTP, dCTP and TTP, and 10 U reverse transcriptase. After 60 min at 42°C, the nucleic acids were collected by ethanol precipitation and centrifugation. The pellets were then analyzed by gel electrophoresis and autoradiography as described above for S1-nuclease mapping.

Construction of Deletion Mutations

The plasmid pSV.OG (Fig. 1b) was made linear by Cla I restriction, and then digested for varying lengths of time with Bal 31 exonuclease (49). The Bal 31 digest contained 200 µg/ml of DNA, 0.6 M NaCl, 12 mM CaCl₂, 12 mM MgCl₂, 0.1 M Tris-HCl, pH 8.0, and 30 U/ml of Bal 31. The reaction was stopped at various times by phenol extraction and ethanol precipitation. Thirty µg of Bal 31 digested DNA were made blunt ended by treatment with E. coli DNA polymerase I (Klenow) in 66 mM Tris-HCl, pH 8.0, 6.6 mM MgCl₂, 1 mM DTT, 66 µM ATP and 0.2 mM each of dATP, dGTP, dCTP and TTP at 12°C for 30 min. Synthetic Cla I linkers [d(CATCGATG):obtained from New England Biolabs], [³²P] end-labelled and phosphorylated as described (50) were added to 2 µM, and ligated with 200 U/ml T4 DNA ligase for 12-16 hrs at 12°C.

The reaction was heated to 68°C for 10 min, made 50 mM in NaCl, and then digested with 100 U of Cla I at 37°C overnight. 50 units of Bgl II were then added and digestion was continued for 4 hrs. The DNA fragments were separated on a 4% polyacrylamide preparative gel and then autoradiographed. DNA fragments migrating faster than 1.25 Kb were eluted from the gel slices (47) and then ligated into the 7.5 Kb Bgl II + Cla I fragment of pSV.OG (Fig. 1b). The ligated DNA was used to transform E. coli RR1 (43) and ampicillin resistant colonies were analyzed by restriction digests of minilysates (46). The deletion endpoints were crudely sized by Bgl II + Cla I digest and agarose gel electrophoresis, and then determined precisely by [³²P] 5' end-labelling at the Cla I site followed by Maxam-Gilbert sequencing (57).

Denaturing Gel Electrophoresis of RNA

RNA was denatured in 8.5 M urea and then electrophoresed on a 2.5% agarose slab gel containing 6 M urea at pH 3.5 in a 50 mM sodium citrate, pH 3.5, running buffer (52). Specific mRNA sequences in the gel were detected by the direct hybridization procedure of Purello and Balazs (53). After electrophoresis, the gel was made free of urea by washing in H₂O, placed on a piece of wet Whatman 3 MM paper and dried at 60°C under vacuum. The gel was prehybridized in a sealed plastic bag for 1 hr at 42°C with a hybridization solution containing 50% formamide, 2 x SSCP (SSCP = 0.15 M NaCl, 0.015 M Na Citrate, 0.025 M phosphate, pH 7.0), 0.1% SDS, 5 x Denhardt's (0.1% Ficoll

400, 0.1% Bovine Serum Albumin, 0.1% polyvinylpyrrolidone), 200 µg/ml of sheared denatured herring sperm DNA and 10 mM EDTA. This solution was then replaced with hybridization solution (minus the herring sperm DNA) containing 10^7 cpm of a heat denatured DNA probe labelled with [32 P] to a high specific activity (2×10^8 cpm/µg) by nick translation (44). Hybridization was at 42°C for 12-18 hrs. The gel was then given two 30-min washes at 65°C in 2 x SSCP and two 30-min washes at 65°C in 0.2 x SSCP before exposure to Kodak XAR-5 film.

RESULTS

Construction of an Ovalbumin β -Globin Hybrid Gene

To study the function of the ovalbumin gene promoter after transfer into cultured cells, we linked the ovalbumin gene's 5'-flanking sequences to the chicken adult β -globin structural gene. This was done because 1) the ovalbumin gene itself is too large to readily manipulate in vitro, and 2) detection of mRNA transcripts initiating at an ovalbumin promoter transferred into chicken oviduct cells requires its linkage to DNA sequences whose transcripts are not normally present in oviduct cells.

The hybrid gene was constructed by ligating a 794-bp Hae III + Taq I fragment from the ovalbumin 5'-flanking region to a 1364-bp fragment of the chicken adult β -globin gene, adding Eco RI linkers, and then cloning into pBR322 (see Methods). The structure of the hybrid gene, called ovalglobin, is shown in Figure 1a. There are 753 bps of ovalbumin 5'-flanking sequence, followed by a hybrid first structural region. The remaining sequences extend to the Hpa II site at +1479 of the chicken adult β -globin gene, within the third structural region (42). The ovalglobin gene thus lacks the globin polyadenylation signal.

For our gene transfer experiments, we cloned the ovalglobin gene into the vector pSV-R1. The vector was constructed by adding Eco RI linkers to a Hpa II + Bam HI fragment of the SV40 genome (4) and cloning into a modified pBR322 plasmid. The modification consisted of removing ~930 bps surrounding the Pvu II site, thereby deleting plasmid sequences which interfere with SV40 driven replication in mammalian cells (54). In the recombinant pSV.0G (Fig. 1b), the ovalglobin gene is cloned in an orientation permitting it to make use of the SV40 polyadenylation signal of the late transcription unit (55). Note also that the SV40 late promoter transcribes in tandem with the ovalglobin promoter.

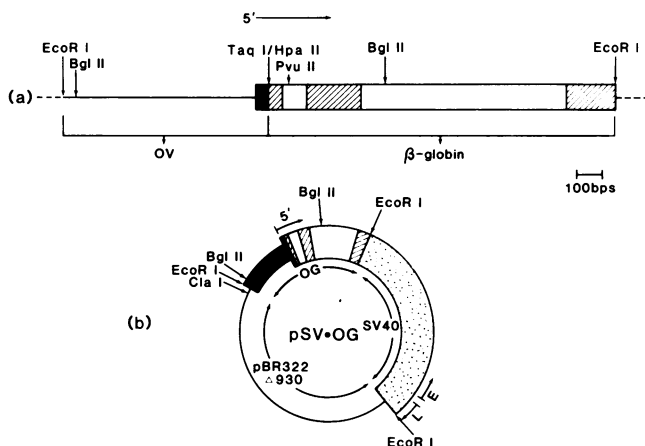


Figure 1: (a) Structure of the ovalglobin gene. Thin line: ovalbumin 5'-flanking sequence; filled box: nucleotides 1-41 of the ovalbumin first exon; hatched boxes: exons of the chicken adult β -globin gene; open boxes: introns of the chicken adult β -globin gene. The Eco RI site at the 5' end is derived from linker addition to the Hae III site at position -753 of the ovalbumin gene. The 3' Eco RI site is derived from linker addition to the Hpa II site at position +1481 of the chicken adult β -globin gene. (b) Structure of the plasmid pSV.OG. Thin line: pBR322 sequences; thick line: ovalbumin gene 5'-flanking sequences; filled box: nucleotides 1-41 of the ovalbumin gene first exon; hatched boxes: exons of the chicken adult β -globin gene; open boxes: introns of the chicken adult β -globin gene; shaded box: SV40 early region. The SV40 sequences extend from the unique Hpa II site (position 282) to the unique Bam HI site (position 2469), both of which were converted to Eco RI sites. The directions of early (E) and late (L) transcription are shown at the lower right.

Correct Splicing of Ovalglobin Gene Transcripts

We transferred the plasmid pSV.OG into HeLa S3 cells by calcium phosphate precipitation and DMSO shock, and then harvested mRNA from the transfected cells 40 hrs later as described in Methods.

To determine whether the primary ovalglobin transcript is accurately spliced, we located the 3' splice sites of both introns by S1-nuclease mapping. In chicken β -globin mRNA, the 3' splice site of intron A is 213 nucleotides 5' of the Ava I site within exon 2 (42). A 1162 bp DNA probe which had been 5' end-labelled with [32 P] on the mRNA template strand at the Ava I site (Fig. 2a) was hybridized to chicken reticulocyte mRNA, digested with S1-nuclease, and then electrophoresed on a denaturing polyacrylamide gel. β -globin mRNA protected a DNA fragment about 213 nucleotides in length, as expected (Fig. 2b, left, lane 3). An identical result was obtained when poly(A)⁺ RNA extracted from pSV.OG transfected cells was hybridized to this

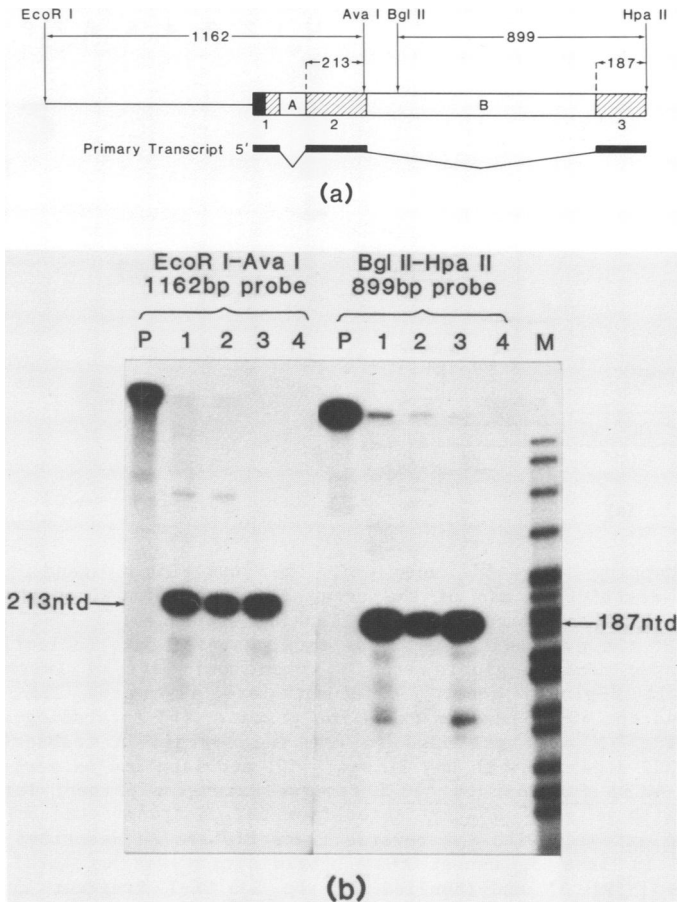


Figure 2: (a) Restriction map of the ovalglobin gene showing the location of probes used to map the 3' splice junctions of introns A and B. The 1162 bp probe was 5' end-labelled at the Ava I end; the 899 bp probe was 5' end-labelled at the Hpa II end. Bold lines underneath represent mRNA sequences present in mature mRNA; thin lines are the excised intronic sequences. (b) S1-nuclease mapping of the 3' splice sites in poly(A)⁺ RNA extracted from chick reticulocytes and pSV.OG transfected HeLa S3 cells. Hybridizations and S1 digests were done as described in Methods, and the products were analyzed on a 4% polyacrylamide/7 M urea gel. Lanes 1 and 2: hybridization to poly(A)⁺ RNA extracted from pSV.OG transfected HeLa cells (two different transfections); lane 3: hybridization to chicken reticulocyte poly(A)⁺ RNA; lane 4: hybridization to total yeast RNA. On the left, the probe was the 1162 Eco RI-Ava I fragment (5' end-labelled on the Ava I end), and on the right, the probe was the 899 bp Bgl II-Hpa II fragment (5' end-labelled on the Hpa II end).

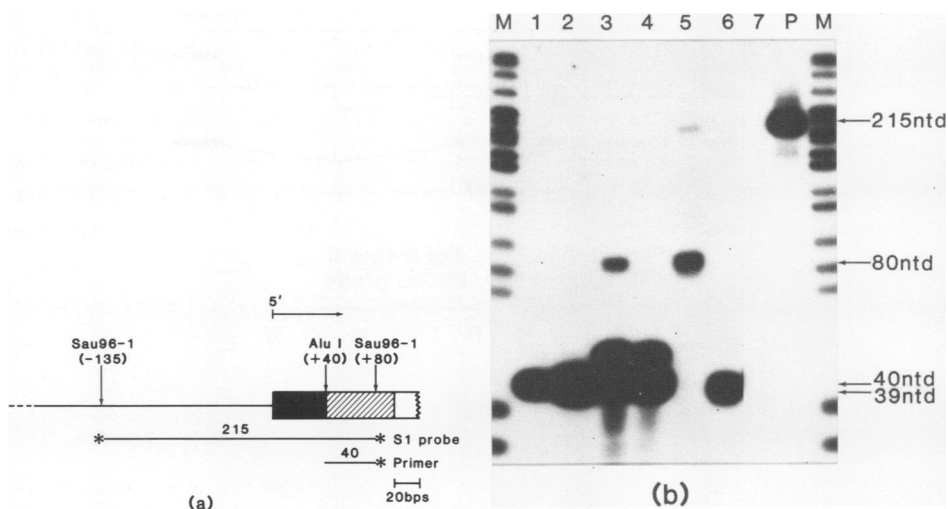


Figure 3: Mapping the 5' ends of the ovalglobin gene transcripts. **(a)** Partial restriction map of the area around the 5' end of the ovalglobin gene. Thin line: ovalbumin gene 5'-flanking sequences; filled box: nucleotides 1-41 of the ovalbumin gene first exon; hatched box: nucleotides 115-170 of the chicken adult β -globin exon 1; open box: part of intron A of the chicken adult β -globin gene. Underneath are shown the 215 bp *Sau* 96-1 S1 probes and the 40-bp reverse extension primer. **(b)** Autoradiogram of the 8% polyacrylamide/7 M urea gel used to map the ovalglobin transcript 5' end. Lane M: pBR322 digested with *Hpa* II and [32 P] end-labelled as markers; lanes 1 and 2: the 40 bp *Sau* 96-1 to *Alu* I reverse extension primer; lane 3: primer hybridized with poly(A)⁺ RNA extracted from pSV.OG transfected HeLa cells and then reverse extended with AMV reverse transcriptase as described in Methods; lane 4: as in lane 3, except that hybridization was to total yeast RNA; lane 5: the [32 P] 5' end-labelled 215 bp *Sau* 96-1 fragment hybridized to poly(A)⁺ RNA extracted from pSV.OG transfected cells and then digested with S1 nuclease as described in Methods; lane 6: as in lane 5, except that hybridization was to poly(A)⁺ chicken reticulocyte RNA; lane 7: as in lane 5, except that hybridization was to total yeast RNA; lane P: migration of the untreated 215 bp *Sau* 96-1 probe.

same *Ava* I probe (Fig. 2b, left, lanes 1 and 2). We mapped the 3' splice site of intron B in a similar way, except that the DNA probe was a fragment 5' end-labelled with [32 P] on the mRNA template strand at the *Hpa* II site near the 3' end of exon 3. This *Hpa* II site is located 187 nucleotides 3' of the 3' splice site of the second intron (Fig. 2a, Ref. 42). Chicken reticulocyte mRNA protected a 187 bp DNA fragment as expected (Fig. 2b, right, lane 3). An identical result was obtained with poly(A)⁺ RNA extracted from pSV.OG transfected HeLa cells (Fig. 2b, right, lanes 1 and 2). No S1-protected products were observed when either probe was hybridized with mRNA extracted from untrans-

ected HeLa S3 cells (not shown). These results indicate that the 3' splice sites (acceptors) of the ovalglobin gene function normally in HeLa S3 cells.

Accurate initiation of the ovalglobin gene in HeLa S3 cells

We used two methods to determine the location of the 5'-end of the ovalglobin transcript: S1-nuclease trimming (56,57) and primer extension. For the S1 experiment, a 215 bp Sau 96-1 fragment extending from -135 to +80 was 5' end-labelled with [³²P]. This fragment was hybridized to poly(A)⁺ RNA extracted from pSV.OG transfected HeLa cells and then treated with S1 nuclease. Electrophoresis and autoradiography of the S1-resistant products revealed a band migrating at about 80 nucleotides (Fig. 3b, lane 5), the position we would expect if ovalglobin transcripts were accurately initiated at the ovalbumin gene cap site (57). The broadness of the band suggests some microheterogeneity, either at the 5' end of the transcript or in the DNA probe. Microheterogeneity of the 5' end of the ovalbumin mRNA has been well documented (58). Also note in Figure 3b, lane 5 that a small amount of probe is protected along its entire length. The intensity of this band varies from one experiment to the next, and is not due to probe reannealing because is not observed after S1 digests of probe annealed to yeast RNA (lane 7). Nor is it due to contaminating DNA, because: 1) when the probe is strand separated, only the isolated coding strand produces an S1-protected band at 215 nucleotides; and 2) when the RNA preparation is treated with alkali prior to hybridization, no S1-protected bands are produced (data not shown). The 215-bp band is most likely due to read through from the SV40 late promoter, although other promoters 3' of the Sau 96-1 site at position -135 might be active.

As a hybridization control, we annealed the 215 bp Sau 96-1 probe to poly(A)⁺ chick reticulocyte RNA. Since the 215 bp [³²P] probe contains β -globin sequences only between the 3' Sau 96-1 site and the Taq I/Hpa II junction (see Fig. 3a), a distance of 39 bps, we expected to see a 39-nucleotide fragment protected from S1 nuclease digestion. As shown in Figure 3b, lane 6, we do indeed see a band migrating at about 39 nucleotides.

The results of S1-nuclease analysis are consistent with two possibilities: 1) the 5' terminus of the ovalglobin transcript is located 80 nucleotides 5' of the Sau 96-1 within exon 1; or 2) there is a splice junction located 80 nucleotides 5' from that same Sau 96-1 site. It is important to rule out this second possibility, because SV40 late leader sequences (generated by the SV40 late promoter) might, by coincidence, be spliced to ovalglobin transcripts 80 bps 5' of the Sau 96-1 site within exon 1 (see Ref. 4). To distinguish these possibilities, we did a primer extension experiment as follows: the 215 bp

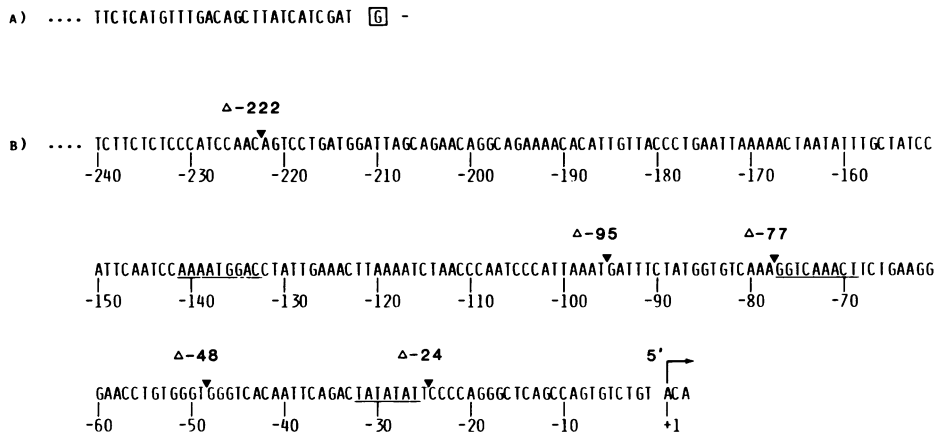


Figure 4: DNA sequence of the ovalbumin gene 5'-flanking region, indicating the locations of deletion mutations used in this study. (a) The pBR322 sequence which immediately precedes the endpoint of each deletion mutation. The boxed guanosine residue is contributed by the *Cla* I linker used in the construction of the deletions. (b) Nucleotide sequence of the ovalbumin gene 5'-flanking region between position -240 and the cap site (+1). The endpoints of the deletion mutations used in this study are indicated by a solid, inverted triangle (∇). Various consensus sequences are underlined: -141 to -133: derived by comparison of the 5'-flanking sequences from steroid inducible chicken genes (68); -77 to -69: a sequence with homology to the "CAAT" box found in many eucaryotic genes; -32 to -26: the "TATA" box.

Sau 96-1 fragment was 5' end-labelled and then digested with Alu I. The 40 bp Alu I-Sau 96-1 fragment was isolated and then hybridized to mRNA isolated from pSV.OG transfected cells. The hybrids were exposed to AMV reverse transcriptase in the presence of all four dNTP's, and the extension product was sized by electrophoresis and autoradiography. If the 5' end of the transcript is located 80 nucleotides 5' of the Sau 96-1 site, then we should observe an 80 nucleotides extension product. If instead there is a splice junction 80 nucleotides 5' from the Sau 96-1 site, we should observe a much longer extension product. As shown in Figure 3b, lane 3, the extension product is indeed 80 nucleotides in length and comigrates with the S1-protected fragment discussed earlier (Fig. 3b, lane 5).

These experiments indicate the presence of unique 5' termini located at the positions consistent with accurate initiation at the ovalbumin cap site of the ovalglobin hybrid gene.

Deletion Analysis of the Ovalglobin Promoter Region

In order to locate the DNA sequences of the ovalglobin gene that are required for transcription initiation, we constructed deletions of varying

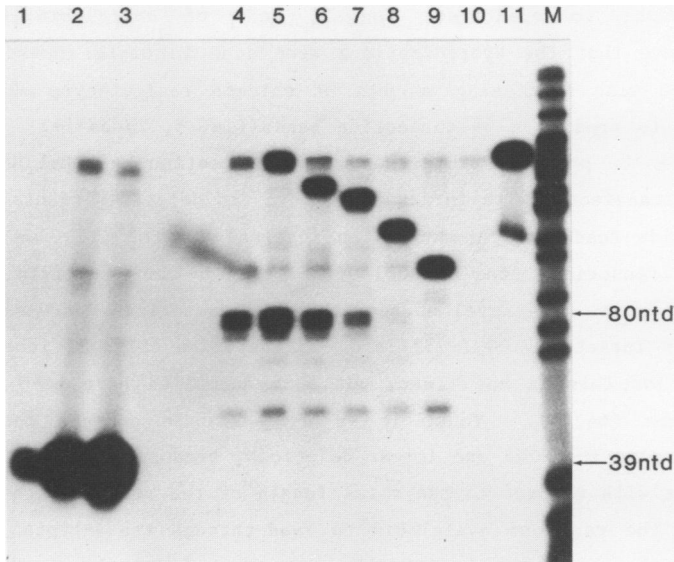


Figure 5: S1 nuclease mapping of ovalglobin transcript 5' ends produced by deletion mutations of pSV.OG transfected into HeLa S3 cells. For each deletion, total RNA extracted from one quarter of a 100 mm dish of transfected HeLa cells was hybridized to 10^6 cpm (30 ng) of the 215 bp Sau 96-1 probe 5' end-labelled with [32 P] and then digested with S1 nuclease as described in Methods. Lane M: pBR322 digested with Hpa II and end-labelled with [32 P] as markers; lanes 1-3: hybridization to chicken reticulocyte poly(A)⁺ RNA (0.01, 0.1 and 1 μ g); lanes 4-9: hybridizations to total RNA extracted from HeLa cells transfected with the intact pSV.OG plasmid (lane 4), the -222 deletion (lane 5), the -95 deletion (lane 6), the -77 deletion (lane 7), the -48 deletion (lane 8) and the -24 deletion (lane 9). Lane 10 is hybridization to total yeast RNA, and lane 11 is migration of the untreated probe. By densitometric scanning of this autoradiogram, we determined the ratios of ovalglobin transcripts (80 nucleotide bands) to read-through transcripts (215 nucleotide bands) to be 4.3 (-753), 0.79 (-222), 0.47 (-95), 0.16 (-77), and 0.015 (-48). No ovalglobin transcripts were detectable with the -24 deletion.

extents within the 5'-flanking region and transfected them into HeLa S3 cells. The deletions were constructed by cleaving pSV.OG with Cla I, digesting for varying times with the double stranded exonuclease Bal 31, and then recloning the deleted fragments as described in Methods. This method of construction insures that each deletion endpoint is joined to exactly the same pBR322 sequences. The deletion endpoints were determined by DNA sequencing and are shown in Figure 4.

Each deletion plasmid was transfected into HeLa S3 cells, and the mRNA from transfected cells was analyzed by S1-nuclease mapping using the 215 bp

Sau 96-1 probe to quantitate the 5' ends of ovalglobin transcripts. To demonstrate that the hybridizations were done in probe excess, the probe was annealed with increasing amounts of chicken reticulocyte mRNA and then S1 digested to produce a 39 nucleotide band (Fig. 5, lanes 1-3). In lanes 4 through 9 are the products protected from S1 digestion by total RNA extracted from cells transfected with intact pSV.OG and its deleted variants. Using the 215 nucleotide read-through band as an internal control (16), we quantitated ovalglobin transcripts (the 80 nucleotide band) by densitometric scanning of the autoradiogram. The level of read-through transcripts increases about 10-fold between intact pSV.OG (-753) and -222 deletion (Fig. 5, lanes 4 and 5). The reason for this is not clear, but such anomalies have been observed in other systems (64,67). Thus, it is necessary to compare the levels of expression among the -222 and longer deletions, because these are all of more similar size with respect to the total length of the read-through transcript. If we make the ratio of ovalglobin to read-through transcripts of the -222 deletion equal to one (Fig 5, lane 5), then the -95 deletion is 0.6 (lane 6), the -77 is 0.2 (lane 7), and the -48 is 0.02 (lane 8). No ovalglobin transcripts were detected with the -24 deletion (Fig. 5, lane 9). Thus, the largest decrease in ovalglobin transcripts results from deletion of the 29-nucleotide sequence between -77 and -48. The region between -77 and -48 contains a sequence resembling the consensus GG_T^CCAATCT, which is found 5' of many other eucaryotic genes at about this location (59). The region between -48 and -24 contains the "TATA box", TATATAT, whose presence is essential for efficient transcription of the ovalbumin gene in vitro (12).

Expression of the Ovalglobin Gene in T47D Cells

Our eventual goal was to achieve regulation of the ovalglobin gene after transfection of hormone-responsive cells. One cell line that we felt might be a particularly good recipient is T47D, derived from a human breast carcinoma. This cell line synthesizes several new proteins in response to estrogen stimulation (60), and contains both estrogen receptor, and large amounts of progesterone receptor (37). We transfected these cells in the presence or absence of 10^{-7} M progesterone, and assayed the level of ovalglobin mRNA 5' ends with the S1-nuclease assay using the 215 bp Sau 96-1 fragment as our probe. It is apparent that progesterone has no effect on the levels of ovalglobin 5' ends (compare lane 1, minus hormone, with lane 2, plus hormone, Fig. 6). This lack of hormone effect is not due to depletion of progesterone receptors as a result of the calcium phosphate or DMSO treatment, because our transfection procedure has no effect on the level of hormone receptors (data not shown).

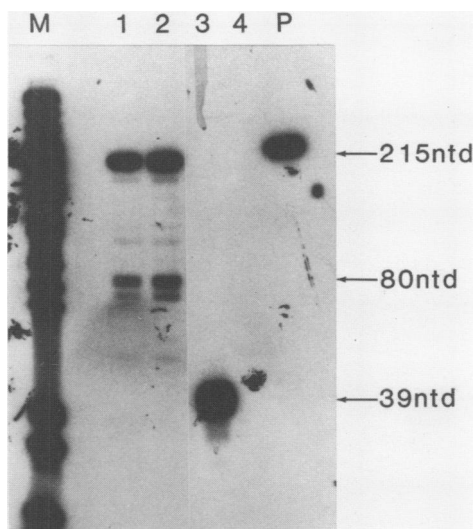


Figure 6: S1-nuclease mapping of the ovalglobin transcripts extracted from pSV.OG transfected T47D cells. Cell culture, transfection and RNA harvest were done as described in Methods. The S1 probe was the coding strand of the 215 bp Sau 96-1 fragment spanning the 5' end of the ovalglobin gene. Lane 1: RNA extracted from cells cultured in the absence of progesterone; lane 2: RNA extracted from cells cultured in the presence of 10^{-7} M progesterone; lane 3: Chick reticulocyte poly(A)⁺ RNA; lane P, migration of the undigested probe; lane M: pBR322 digested with Hpa II and end-labelled with [³²P].

We also attempted to increase ovalglobin transcription with estrogen, estrogen plus progesterone, and a progesterone analogue (R5020) which is more stable than progesterone in the culture medium (K. Horwitz, personal communication), all without effect (data not shown).

Expression of the Ovalglobin Gene in Transfected Chick Oviduct Cells

In order to study the regulation of the ovalglobin gene by progesterone, we transfected the plasmid pSV.OG into primary oviduct cells. These are cultures of nondividing epithelial cells which retain normal amounts of estrogen and progesterone receptors (36).

Oviduct cultures were maintained in the absence of hormone for 4 days, and then transfected by calcium phosphate coprecipitation. One half of the transfected cultures were re-fed medium without hormone and the other half received 10^{-7} M progesterone. After 48 hrs, mRNA was extracted from the cultures and analyzed by Northern gels as described in Methods, using as probe the 1364 bp β -globin Hpa II fragment. In Figure 7 (lanes 3 and 4), ovalglobin transcripts are detected among the transfected cell mRNA. Further, the

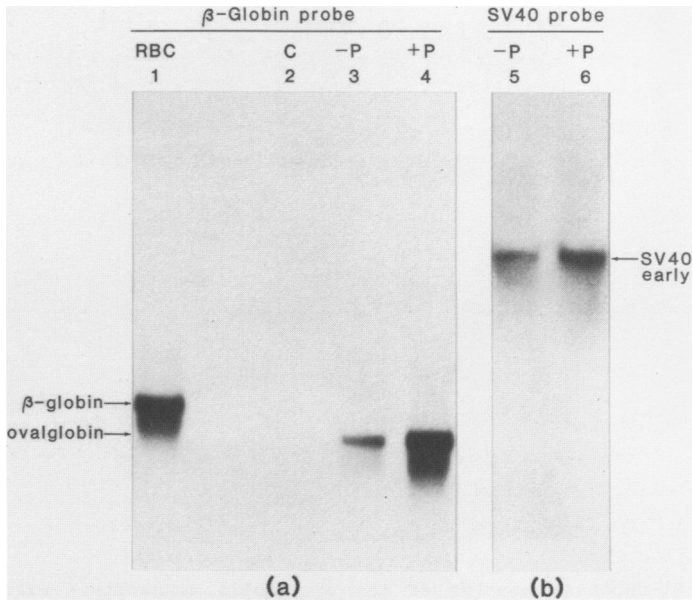


Figure 7: Expression of the ovalglobin and SV40 early genes in pSV.OG transfected primary chicken oviduct cells. Primary oviduct cultures were transfected with pSV.OG as described in Methods and then cultured in the presence or absence of 10^{-7} M progesterone for 48 hr before RNA harvest. RNA was electrophoresed on a 2.5% agarose acid/urea gel, and then hybridized *in situ* to [32 P] labelled DNA probes. Lane 1: 5 ng chicken reticulocyte poly(A) $^{+}$ RNA; 2: 40 μ g poly(A) $^{+}$ RNA extracted from untransfected oviduct primary cultures; lanes 3-6: 40 μ g poly(A) $^{+}$ RNA extracted from pSV.OG transfected primary oviduct cultures; lane 3 and 4: hybridization to [32 P] labelled β -globin probe (the 1364 bp Hpa II fragment); lanes 5 and 6: hybridization to a [32 P] labelled SV40 early region probe (the 1097 bp Dde I fragment); lanes 4 and 6: transfections in the presence of 10^{-7} M progesterone; lanes 3 and 5: transfection without added hormones. In ten different experiments, the level of SV40 early transcripts in pSV.OG transfected oviduct cells cultured in the presence of progesterone averaged about 1.02 times that present in transfected cultures not treated with hormone.

addition of 10^{-7} M progesterone causes an increase in the level of ovalglobin transcripts (lane 4). As a control, this same gel was washed free of [32 P] and rehybridized to a [32 P]-labelled SV40 probe (the 1097 bp Dde I fragment) to detect SV40 early transcripts. These transcripts appear to be present in equivalent amounts in the presence or absence of 10^{-7} M progesterone since in 10 separate experiments, the average induction ratio for the SV40 early transcript was 1.02, as determined by densitometry of the autoradiograms. This result is in agreement with that of Renkawitz *et al.* (24), who observed no

effect of progesterone on SV40 early gene expression after microinjection of SV40 DNA into cultured oviduct cells. Densitometry of this autoradiogram indicates a >10-fold induction of ovalglobin transcripts by 10^{-7} M progesterone, normalized to the nonhormonally responsive SV40 early transcript. In five independent experiments, the normalized induction ranged from 3- to >10-fold.

DISCUSSION

These studies were intended to investigate the role of 5'-flanking sequences in the promotion of transcription of the ovalbumin gene. We anticipated that important control sequences would be present near the 5' end of the ovalbumin gene because it contains two areas of homology with the 5'-flanking regions of many other eucaryotic genes: the "TATA" box at position -24 to -32 (TATATAT), and a sequence corresponding to the "CAAT" box at position -69 to -77 (CCTCAAAC) (59).

To carry out these studies, we constructed a hybrid gene in which the ovalbumin 5'-flanking region was joined to the chicken adult β -globin gene. The hybrid gene is small, thus facilitating the construction of deletions, and also contains several restriction sites within the first exon suitable for S1-nuclease analysis and primer extension. Most importantly, the ovalbumin promoter is joined to sequences whose transcripts can be readily detected among mRNA extracted from transfected oviduct primary cultures. The ovalglobin hybrid gene was then joined to SV40-pBR322 sequences, providing for gene amplification after transfection of HeLa cells, and also a polyadenylation site for the ovalglobin gene. The transcription of the SV40 early region also serves as an internal control on the expression of the ovalglobin gene in transfections of oviduct cells. It is also possible that the SV40 72 bp repeats ("enhancers") are necessary for ovalglobin transcription in HeLa cells, as they are for the transcription of several other genes that have been tested (6,61), but we have not yet determined this directly.

Transfection of HeLa S3 cells results in ovalglobin transcripts whose 3' splice sites are identical (or nearly so) to those of mature β -globin mRNA (Fig. 3b). We assume that these 3' splice sites are joined to the same 5' splice sites as in β -globin mRNA, but we have no direct evidence for this. However, the 3' splice site of intron B must join to some sequence 3' of the Ava I site in exon 2, since we can readily detect this Ava I site in mRNA extracted from transfected HeLa cells. Similarly, the 3' splice site of intron A must join to some sequence 3' of the Sau 96-1 site in exon 1.

In addition to being accurately spliced, these ovalglobin transcripts also

appear to initiate accurately in transfected HeLa cells at or near the ovalbumin gene cap site. We determined this by two methods: S1-nuclease trimming and primer extension. This observation has permitted us to generally define the extent of 5'-flanking sequences needed for promoting the initiation of ovalbumin gene transcription. The results of our deletion mapping experiment (Fig. 5) must be considered semiquantitative, because the levels of read-through transcripts that we used as our internal control may be affected by the deletions in the 5'-flanking of the ovalglobin gene. With this caution in mind, it is clear that deletion of a very small number of nucleotides (between -77 and -48) causes the largest decrease in ovalglobin transcripts (Fig. 5, lanes 7 and 8). In contrast, in vitro transcription of the ovalbumin gene requires only DNA sequences up to -44 (8). This difference could be due to physical differences between the linear templates used in in vitro transcription reactions and the circular, supercoiled DNA replicating in transfected cells (14,62). It is also possible that the different sequence requirements observed in vitro and in vivo are due to the presence of viral enhancer elements on the transfected DNA molecules in vivo.

Our data do not allow us to further define the extent or internal structure of the ovalbumin promoter region. However, we would expect that the sequence TATATAT (-24 to -32) is an important element of the promoter, because a point mutation to TGTATAT greatly reduces transcription of the ovalbumin gene in vitro (12). Also, in studies with the rabbit β -globin gene (15,16), HSV TK gene (18,19) and conalbumin gene (63), the TATA sequence is necessary for efficient transcription in vivo. In addition to the TATA sequence, the ovalbumin promoter must contain other elements, located 3' of the -77 deletion. It is likely that one element is the sequence 3'GGTCAAAC-5' (-77 to -69), which resembles a consensus sequence (GG_T^C CAATCT) found in a similar position in many other eucaryotic genes (59). This sequence is necessary for efficient transcription of the rabbit β -globin gene (15,16), Drosophila Hsp 70 gene (20), and human α 1-globin gene (64) in transient transformation experiments. Further, a naturally occurring base substitution in this sequence upstream from the human adult β -globin gene causes a marked decline in the transcription of the gene when it is transferred into HeLa cells (13). However, in several other cases (SV40 early promoter [65] and HSV-TK [15,16]), this upstream consensus sequence is not required for efficient transcription. Our experiments suggest that the ovalbumin gene promoter may require the GG_T^C CAATCT consensus sequence for efficient transcription, but more precise mutations will have to be analyzed to prove this.

While these studies have helped to define the basal ovalbumin gene promoter as it functions in HeLa cells, we were also interested in determining whether there are 5'-flanking DNA sequences involved in the hormonal regulation of the ovalbumin gene. We first attempted to achieve regulation of the ovalglobin gene by transferring it into T47D cells, which contain large numbers of progesterone receptors. The ovalbumin promoter functioned correctly in these cells, but neither progesterone or estrogen stimulated an increase in ovalglobin transcripts. This lack of induction could be due to a failure of heterologous receptors or regulatory molecules to recognize ovalbumin regulatory sequences, or to a dilution of regulatory factors by large numbers of ovalglobin gene copies in transfected cells. In a separate study, when the ovalbumin gene was stably integrated at low copy number into the genome of estrogen-responsive MCF7 cells (60), it was induced about 6- to 10-fold by estrogen (Lai, Riser and O'Malley, submitted).

With the development of a chicken oviduct cell culture system (36), we were able to attempt the transfer of the ovalglobin gene into chicken oviduct cells. Using a highly sensitive gel hybridization procedure, we detected ovalglobin transcripts in cells transfected with pSV.0G by standard calcium phosphate coprecipitation. The level of ovalglobin transcripts increased when progesterone was added to the medium, indicating that the transfected gene was regulated in these primary oviduct cells. In contrast, progesterone had no effect on the level of SV40 early transcription.

We have used this assay to determine which 5'-flanking sequences are necessary for the induction of ovalglobin transcription by progesterone. These studies indicate that DNA sequences between -95 and -222 are necessary for the efficient induction of ovalglobin transcription by progesterone (66). On the other hand, we have shown that deletion to -95 has little effect on ovalbumin promoter function in transfected HeLa cells, compared with the -222 deletion (Fig. 5). Thus, it appears that elements of the ovalbumin gene promoter are separate and structurally distinct from those of the hormone responsive regulatory region.

It is difficult to estimate the rate at which individual ovalglobin templates are being transcribed, or the numbers of DNA molecules entering transfected cells. These estimates are difficult to obtain because we do not know the efficiency of transfection. Renkawitz *et al.* (24) reported that $<10^{-4}$ cultured oviduct cells transfected with SV40 DNA were positive for T-antigen synthesis by immunofluorescence, but our studies cannot be compared directly because their immunofluorescence assay may not be sensitive enough to

detect cells making small amounts of T-antigen.

Our experiments and those of Renkawitz et al. (24) are the first to employ gene transfer into primary cell cultures. We believe that this approach will prove most valuable in the study of the regulation of specialized genes in highly differentiated tissues. Past studies using heterologous combinations of genes and continuous cell lines have yielded much information about general aspects of gene function. Future studies using promoter-gene fusions (such as ovalglobin) and primary cultured cells will reveal how specialized genes work in their native differentiated cells.

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